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INACTIVATION AND STABILIZATION OF THROMBIN.

WITH 14. FIG. IN TEXT.

BY DR M. GERENDÁS.

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(RECEIVED FOR PUBLICATION 17. 7. 47.)

It is well known from the literature on blood-clotting that in that process thrombin disappears from the blood shortly after the clotting is ended. It loses its effect much more slowely in purified thrombin solutions, but also in a relatively short time. Barrat[1] had already established through his investigations by 1932 that the activity of thrombin solutions deceased also in sterile conditions; therefore its destruction is not caused by fungi or bacteria.

My investigations carried out with purified thrombin solutions also proved that filtration with a Seitz-filter and subsequent maintenance of sterile conditions was not enough to inhibit the inactivation.

Attemps to conserve it with antiseptics can be divided into three groups, according their effects:

1. The activity of thrombin ceases immediately:
   Chlorozon 1‰, potassiumhypermanganate 0,5%, formalin 1%, Lysol 0,5%. Lysochlore 0,5%, Hyperol 3%, sublimate 0,5‰.
2. Activity decreases 50% in 8—10 days:
   Boracic acid 3%, Carbolic acid 3%, Trikresol 2‰, Nipagin-Nipasol 2,5—2,5‰, Ethylalcohol 40%.
3. 50% decrease in activity after 50 days:
   Chinosol (8-oxychinolin-potassiumsulfate) 1‰, Acrigonin 1‰.

Control solutions containing no antiseptics were inactive within 2—3 days. Bacteria can be considered as primarily responsible. (Figure 1)

The antiseptics of the first group obviously acted chemically on the thrombin and stopped its activity. (Laki uses this effect of permanganate in investigation of the kinetics of thrombin activity to interrupt the reaction.)[2]

The second group leaves the thrombin effect untouched and makes the solution bacteria-free, but in spite of this it cannot prevent the thrombin from slowely losing its activity from some other cause.

The stablizing effect of the third group is remarkably good, hence it must be supposed that the compounds in question have some specific capacity beyond their bactericide effect through which they can inhibit the inactivation of thrombin.

It can be stated beyond question from the experiments that in the stabilization of thrombin solutions freedom from bacteria is not enough and that we must also prevent thrombin inactivation. The behaviour of the chinosol thrombin solution provided the first that the inactivation can be inhibited by chemical means.

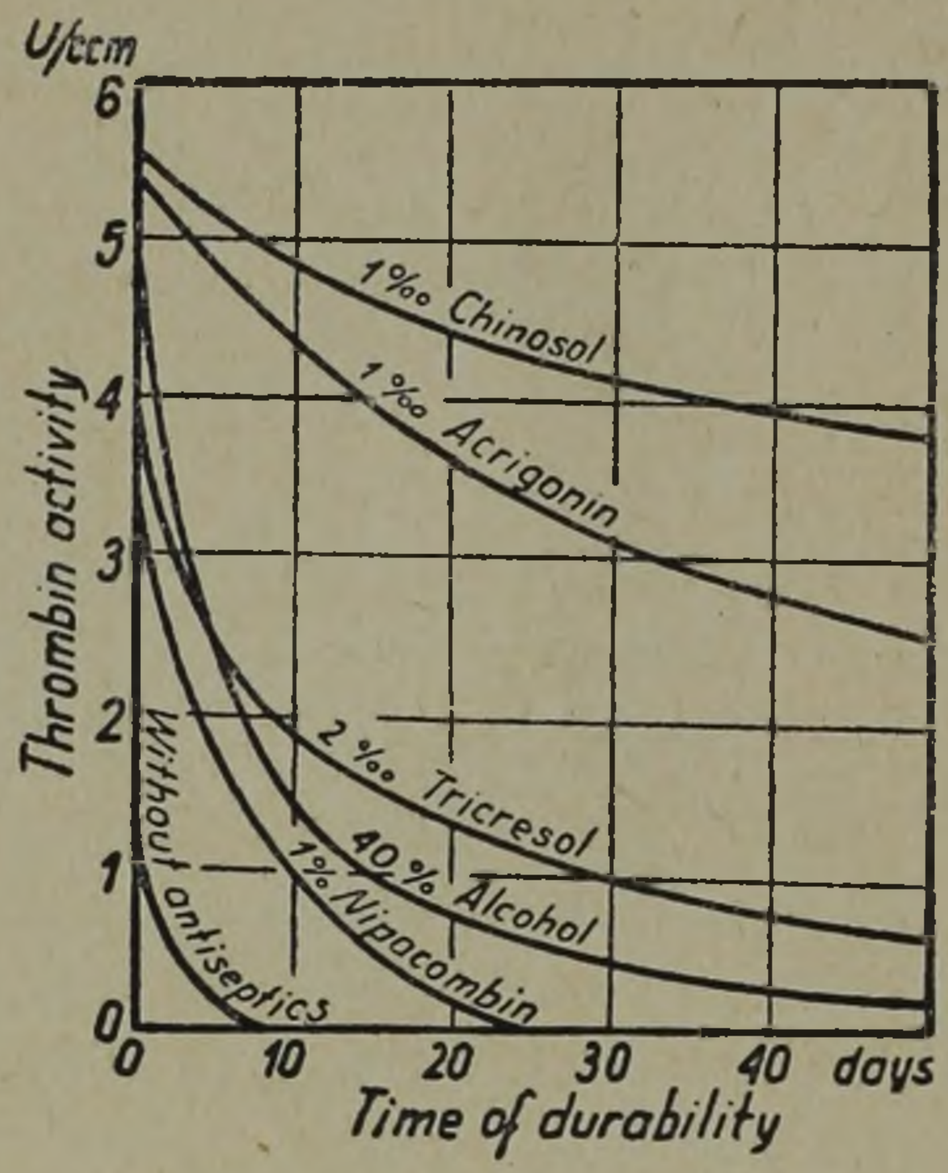

Figure 1.

CHANGES IN THROMBIN ACTIVITY IN NATIVE BLOOD.

If we examine the beginning of the clotting process in fresh blood, that is, the amount of thrombin disappearing, it can be established that the value of the thrombin activity at the beginning of clotting, cca in the 4^{th} minute, is about 1,5—2,0 U/ml, which increases further and in the $7—8^{th}$ minute, strictly speaking, after the completion of the clotting, value reaches the amximum of 3—4 U/ml (Figure 2).

For the experiment 2 ml native blood or recalcified oxalate blood was used. From this blood I took samples at 1-minute intervales, put them into fibrinogen and determined the clotting time. I prepared the fibrinogen by Laki's method.[3] I put the mixture used for determining the clotting time in the bottom of a „Jena" No. 5250 glass dish and established the instant of beginning of clotting with a glass hook. I used the following mixture:

0,1 ml fibrinogen solution (10 mg/ml fibrinogen)
0,1 „ 0,7% NaCl solution
0,1 „ mol/5 phosphate buffer (pH = 7,0)
0,1 „ juice pressed from the clots ..... 1)

From the clotting time the amount of thrombin can be established and expressed in units. 1 thrombin unit is the amount of thrombin which clots 1 ml blood in 1 min. at 20 C°. As the relation between thrombin activity and the clotting time is not linear, I proceeded by establishing experimentally the relation between clotting time and thrombin activity, and took the necessary data in each case from the curve based on the results (Figure 3). For the experiments always cattle blood was used.

From Figure 2, it is also apparent that after clotting the thrombin disappears relatively quickly from the blood, its activity diminishing in 1 hour to $1/10^{th}$ of the maximum value. Sharply distinguishable on the curve are the phases of thrombin formation and thrombin disappea-

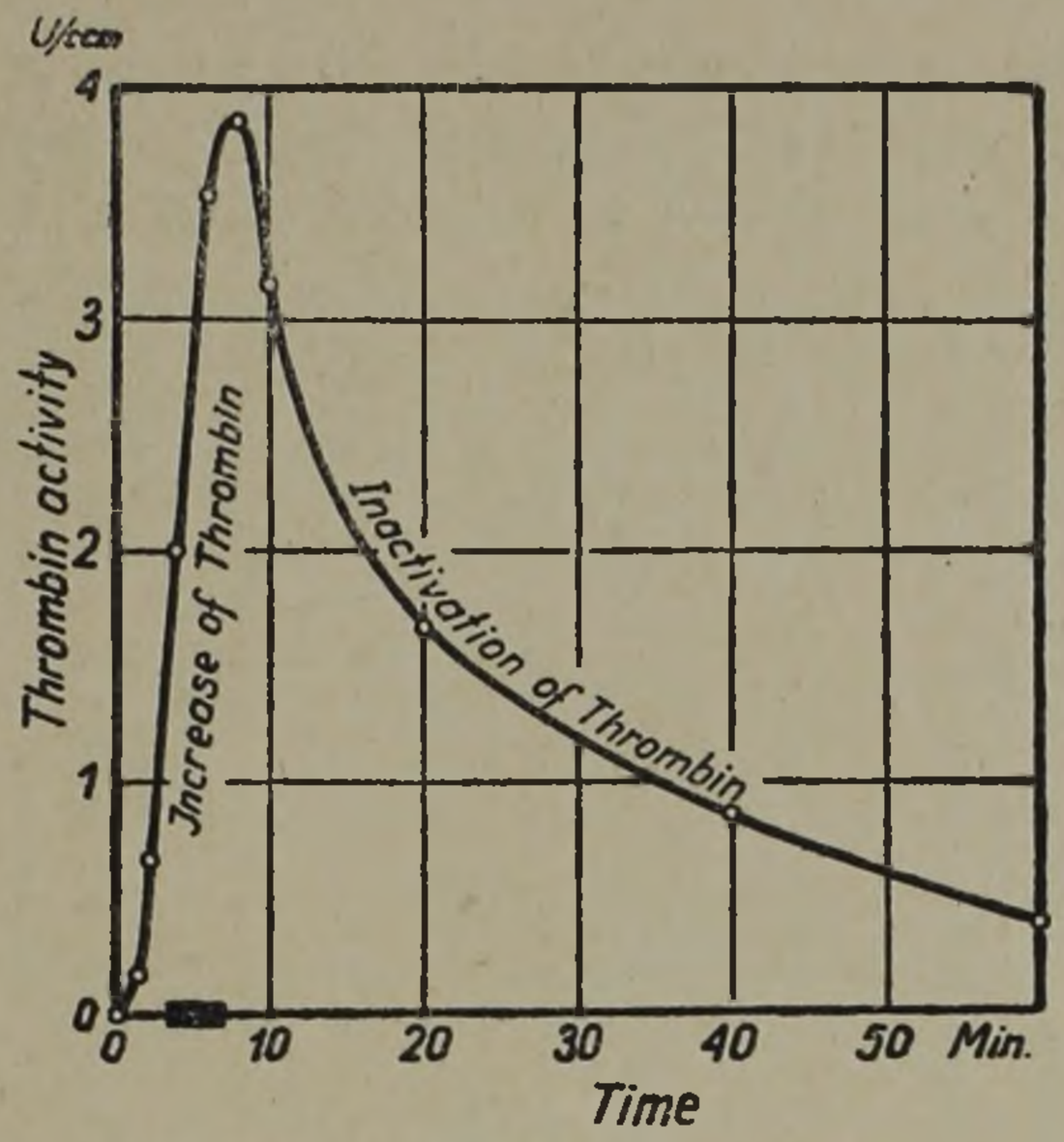

Figure 2.

rance. The picture obtained in this way of the amount of thrombin is, however, entirely false, for the amount of thrombin actually forming, as shown in the Figure, is definitely greater, but in consequence of the inactivating effect it cannot rise above a value of 3—4 U/ml.

If we are to get a correct picture of the phenomenon the two process must be separated. Given suitable experimental conditions (see below, p. 113. estimation of the plasma's real thrombin content), the inactivating process can be eliminated and the formation of the thrombin can be followed without interference. The inactivating effect can easily

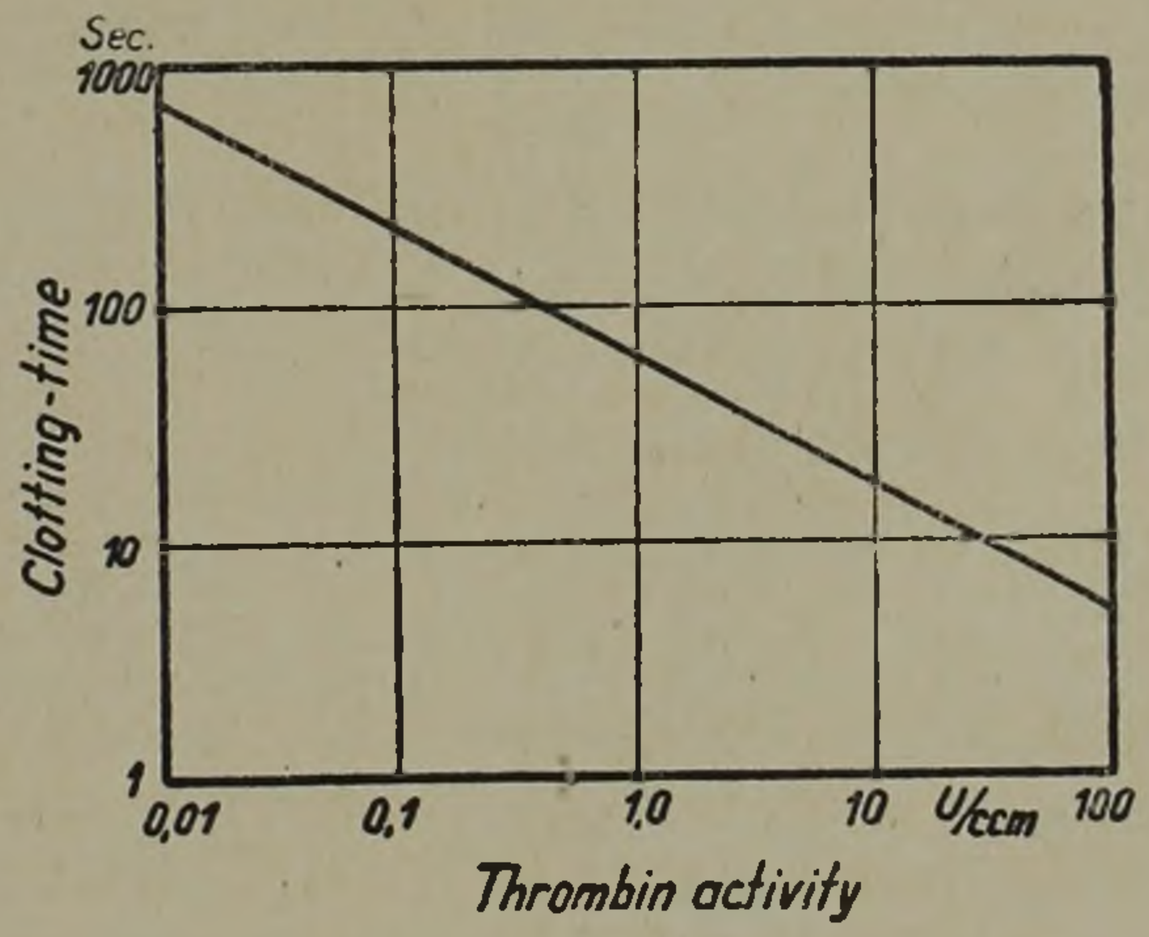

Figure 3.

be examined if we put thrombin into blood or serum. It can be demonstrated that the activity of 100 units of thrombin diminishes in a few minutes to 3—4 units (Figure 4.) following addition to 1 ml blood.

For investigating thrombin inactivation the following experiment is very useful: To 1 ml serum I added 100 units purified thrombin and at different intervals determined the amount of thrombin still present (Figure 4., Curve I.). Repeating the addition of thrombin, a similar rapid inactivation is perceptible (Curve II.). Exhaustion of the inactivation is not apparent, therefore, after the addition of such doses of thrombin.

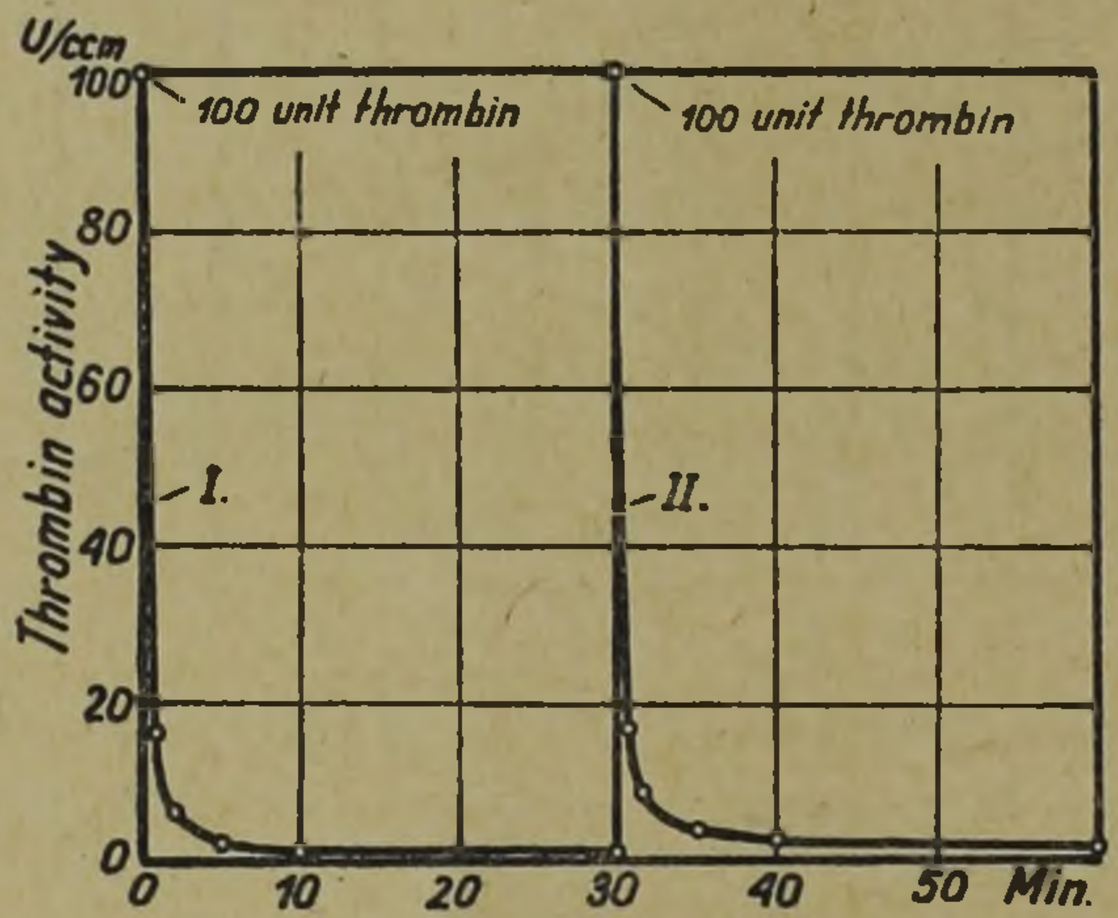

Figure 4.

The foregoing proves that in clotting there can be demonstrated two simultaneous, opposite processes, a thrombin value of 3—4 U/ml being the result of formation and inactivation.

DECREASE OF INACTIVATING EFFECT DURING PURIFICATION OF THROMBIN.

The first step in the isolation of thrombin is the precipitation of prothrombin from diluted plasma with acetic acid. It can be demonstrated experimentally that in this process the greatest part of the inactivating substance remains in the diluted plasma.

If we compare the thrombin inactivating capacity of oxalate plasma (Figure 5, Curve a) with the inactivating effect of diluted prothrombin precipitate (Figure 5, Curve b), the latter's inactivating capacity is seen to be decidedly weaker than that of the plasma. The solution prepared from thrombin powder dried with acetone has still less inactivating capacity (Figure 5, Curve c).

It appears from comparison of the curves that during purification of thrombin we get rid of a considerable part of the inactivating substance, though some of it is carried on, as an impurity, in the prepara-

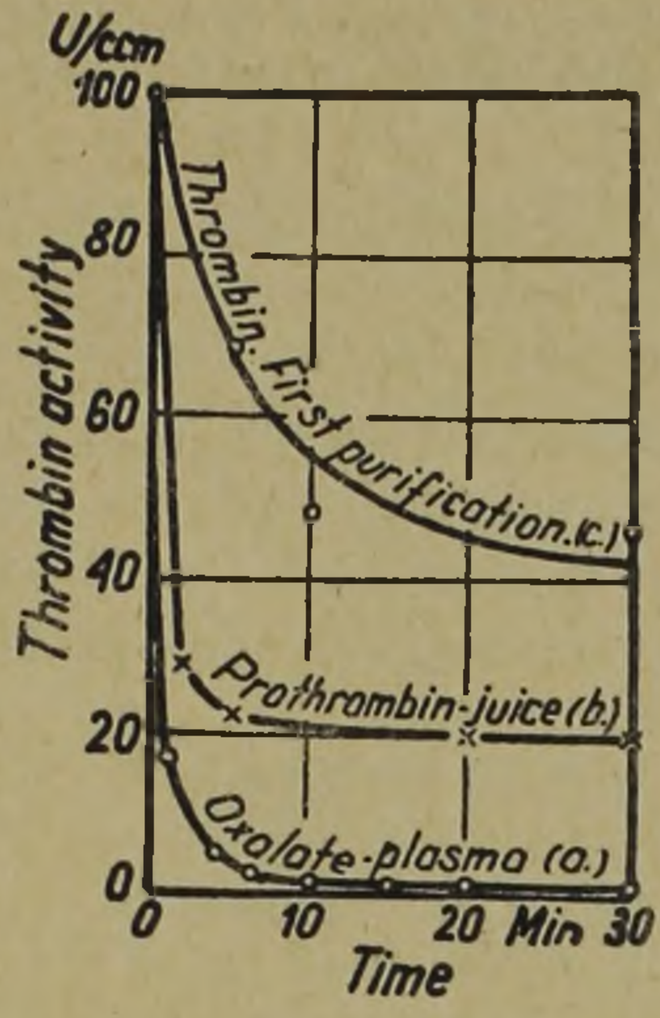

Figure 5.

tion. From the foregoing it is beyond doubt that the inactivation of purified thrombin powders (and solutions prepared from them) which occurs in the course of time is caused by the same inactivating system which, in native blood, in plasma, or in serum, also leads to disappearance of thrombin. From the standpoint of stabilizing thrombin preparations it is very important to clear up the nature of inactivation. In this way we can obtain a process with which to free the thrombin solution of the inactivating impurity, that is, we can find the substances causing the inactivating effect.

LITERARY AND CRITICAL VIEW OF THROMBIN INACTIVATION (TRANSFORMATION TO METATHROMBIN).

Thrombin inactivation is dealt with in the literature under the name of transformation to metathrombin, though so far no unanimous opinion has been formed of the mechanism of the process.

According to Moravitz,[4] the decrease in activity of thrombin present in the serum after clotting can be attributed to the formation of an ineffective substance-metathrombin. Rettger advances the opinion that the spontaneous disappearance of the thrombin from the serum is based on some connection of thrombin to plasma proteins. According, to Hedin,[6] Landsteiner,[7] and Landsberg,[9] thrombin inactivated by combining with the serum albumin. The binding, however, according to Hedin and Landsberg differs from ordinary adsorption, inasmuch as its binding strength increases with a rise in temperature (which is just the contrary of a characteristic adsorption). Furthermore, Lenggenhager and Quick[10] find that inactivation on serum and serum-albumin depends to an equally great extent on the duration of contact (which again is opposed to our knowledge of adsorption). On the other hand, Pekelharing[11] considers the serum inactivating substance at least partly dialysable, Moravitz[12] com-

municates data to the effect that the anti-thrombin is lost by dialysis. As the serum albumin does not disappear with dialysis, this observation stands in decided opposition to the adsorption hypothesis.

Thus the literary data consider the inactivation of thrombin partly as adsorption, partly as the effect of some dialysable substance. To clear up and compare these mutually contradicting observations, I showed in the course of my observations that inactivation of thrombin is neither due to adsorption alone, nor exclusively to the effect of some dialysable substance, but that it is composed of the two processes, a sudden adsorption and a fermentative inactivating process.

INVESTIGATION OF THE PROCESS OF THE DISAPPEARANCE OF THROMBIN.

For the experiment I used serum pressed from clotted blood after letting it stand for 1 hour, when the thrombin formed during clotting has, practically speaking, disappeared. Using to 1 ml serum 16 mg 6,3 u/mg (altogether 100 u) in a way similar to the experiment shown in Figure 3., I determined the curve of thrombin disappearance. By calculation of the kinetics of the reaction it was established (Figure 6.) that, calculating after the monomolecular reaction type, the process gives a constant k-value.

TABLE I.

Thrombin inactivation in serum. Kinetic reckoning.

$$k = \frac{l}{t} . \; 2{,}3 \; . \; log \frac{a}{a-x} \qquad 26\,C^{\circ}$$

Time min.	Clotting time secs.	U/ml	a	a—x	t (min)	k
0	—	100	—	—	—	—
¼	10,5	26,5	100	26,5	¼	5,4
½	11,5	22,7	26,5	22,7	¼	0,59
1	13,0	17,9	22,7	17,9	½	0,48
2	16,5	11,5	17,9	11,5	1	0,44
4	26	5,1	11,5	5,1	2	0,42
6	42	2,05	5,1	2,05	2	0,46
10	105	0,38	2,05	0,38	4	0,42

The k-values calculated from the data (the last column of the Table) aggree satisfactorily, only the figure obtained for the first interval gives a 10-times greater value than the others. This difference exceeds far beyond the limits of experimental error and calls attention to the fact that the thrombin concentration diminishes in the beginning in consequence of some other process. It appeared in the course of further investigations that the rapid decline of the thrombin occuring in the beginning is a consequence of the adsorption of a part of the thrombin.

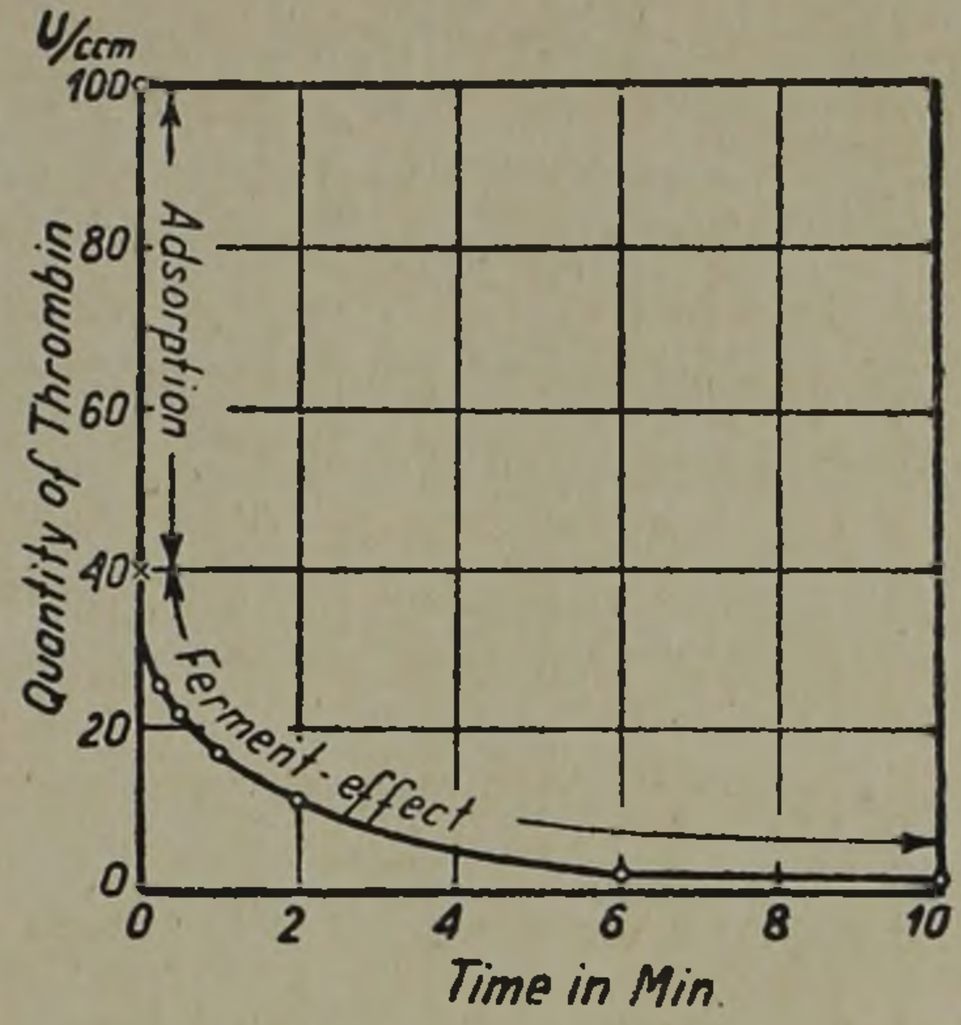

Figure 6.

If we solve the question of reaction speed in respect to a putting a value of 0,5 in place of the k-data of the first interval, the amount of adsorbed thrombin can also be established:

$$a = (a\text{-}x) : N \log \frac{k \cdot t}{2{,}3} = 30 \ U/ml$$

which means that 70% of the thrombin is adsorbed.

It follows from the experiments that in the decline of thrombin activity a rapid adsorption and a constantly inactivating-process play a role. The above observation is in complete harmony with apparently contradictory results of earlier investigators, a part of which attributed the inactivation to adsorption, while others to the effect on a dialysable material. According to my results, both effects play an equal role in diminishing the thrombin value. In the following chapters I give the details, in respect to adsorption and inactivation.

ADSORPTION OF THROMBIN. (ADSORBING EFFECT.)

The curve of the inactivation shown in Figure 6, is constructed from data given in the previous chapter, as the result of two processes. These two processes can also be separated by heating the serum. If the serum is heated to 65—70 C° the inactivator in it loses its effect, while the adsorptive capacity, however, remains unchanged (Figure 7).

Experimental method: 5 ml serum was kept for 10 minutes at the desired temperature and stirred, then cooled to room-temperature (20 C°). (Below 50 C° the serum remains unchanged; above 50 C° it becomes turbid, that is, coagulation takes place. Therefore after cooling I used these tests sieved or pressed out.)

Control	Serum
1 ml distilled water	1 ml warmed serum, re-cooled to 20 C°
0,1 „ thrombin solution	0,1 „ thrombin solution..... 3.

After adding the thrombin, samples were examined at intervals of 1, 2, 5 and 10 minutes, in the following combination:

fibrinogen solution	0,1 ml
test-juice	0,1 „ ..... 4.

With the thrombin put into distilled water as control value thrombin strength of 12 U/ml needed for clotting in 16 seconds forms the point of departure for the series of curves; the values obtained with the serum give the curves themselves (Figure 7.).

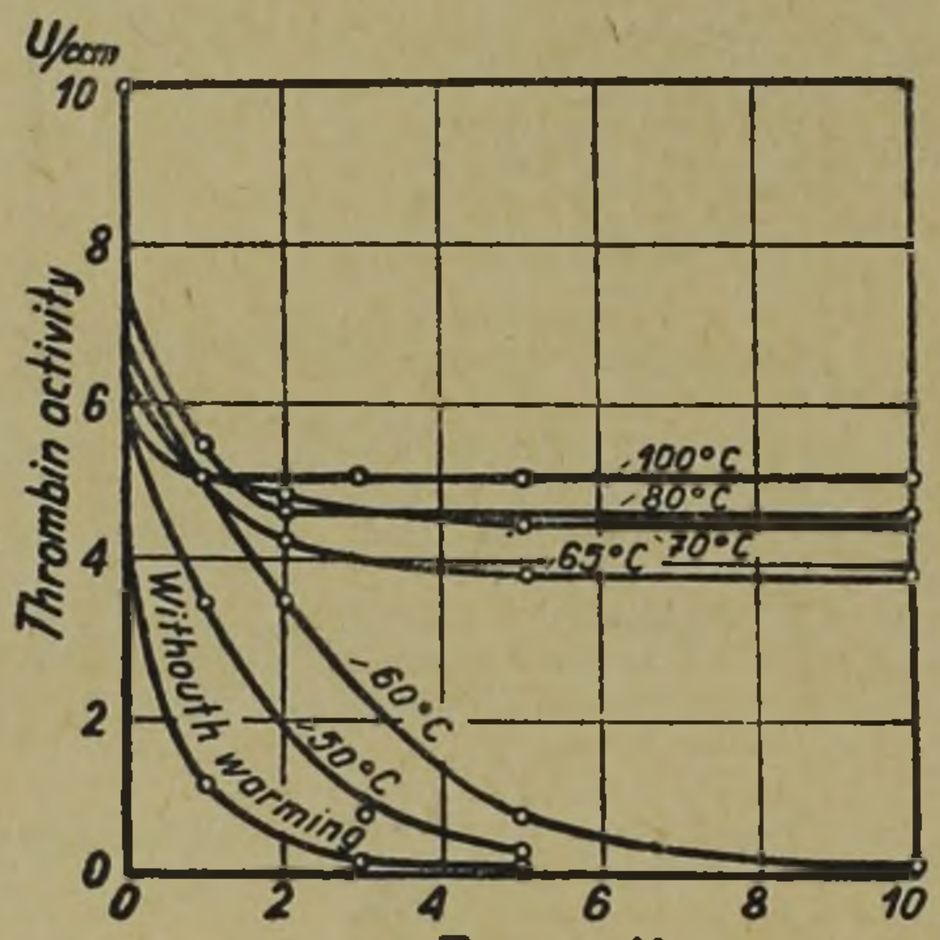

Figure 7.

It is apparent from the curves that the destruction of the inactivator takes place gradually. At around 65—70 C° the effect is already entirely abolished, which means that the curves become straight and horizontal. Thrombin activity however, falls also in these sera to a constant value (about half of the original), which must be accounted far by adsorption. To decide whether the elimination of the coagulum brought about by heating, influences adsorption, I examined filtered and unfiltered serum kept at 60 C°. The result was the same in both cases, hence adsorption takes place in consequence, not on the coagulum, but on some substance remaining in the serum.

OBSERVATIONS ON THROMBIN ADSORPTION.

According to the above, if we heat serum to more than 80 C°, and cool it again, it loses its thrombin inactivating capacity, its adsorbing capacity, however, remains unchanged. In such sera the course of thrombin adsorption can be examined. Adding thrombin of known activity serum thus prepared, the amount of free thrombin can be deter-

mined in clotting experiments with samples taken from the serum, the amount of adsorbed thrombin from the difference between the added and the free thrombin. Carrying out the experiment with thrombin, the course of which closely follows the Langmuir formula.

Experimental data: Serum kept for 10 minutes at 80 C° is pressed out from the coagulum formed as effect of the heating, and cooled to 20 C°. Then in parallel experiments, on the one hand in distilled water, on the other in the prepared serum, we put thrombin solutions of gradually increasing concentrations.

1 ml distilled water		1 ml serum	
0,1 „ thrombin solution	5.	0,1 „ thrombin solution	6.

After mixing we examined, with fibrinogen, the activity of samples taken from the mixture:

0,1 ml fibrinogen	
0,1 „ sample of juice	7.

On the basis of clotting time obtained from the distilled water tests, the amount of thrombin added can be determined, from the serum tests prevailing thrombin concentration. The difference between the two is the amount of thrombin adsorbed.

TABLE II.

Thrombin adsorption in serum.

Thrombin added	U/ml	100	85	70	50	30	15	7,5	4	2
Free thrombin	U/ml	54	43	34	21	10	4,3	2,1	1,5	0,44
Adsorbed thrombin ...	U/ml	46	42	36	29	20	10,7	5,4	2,95	1,56

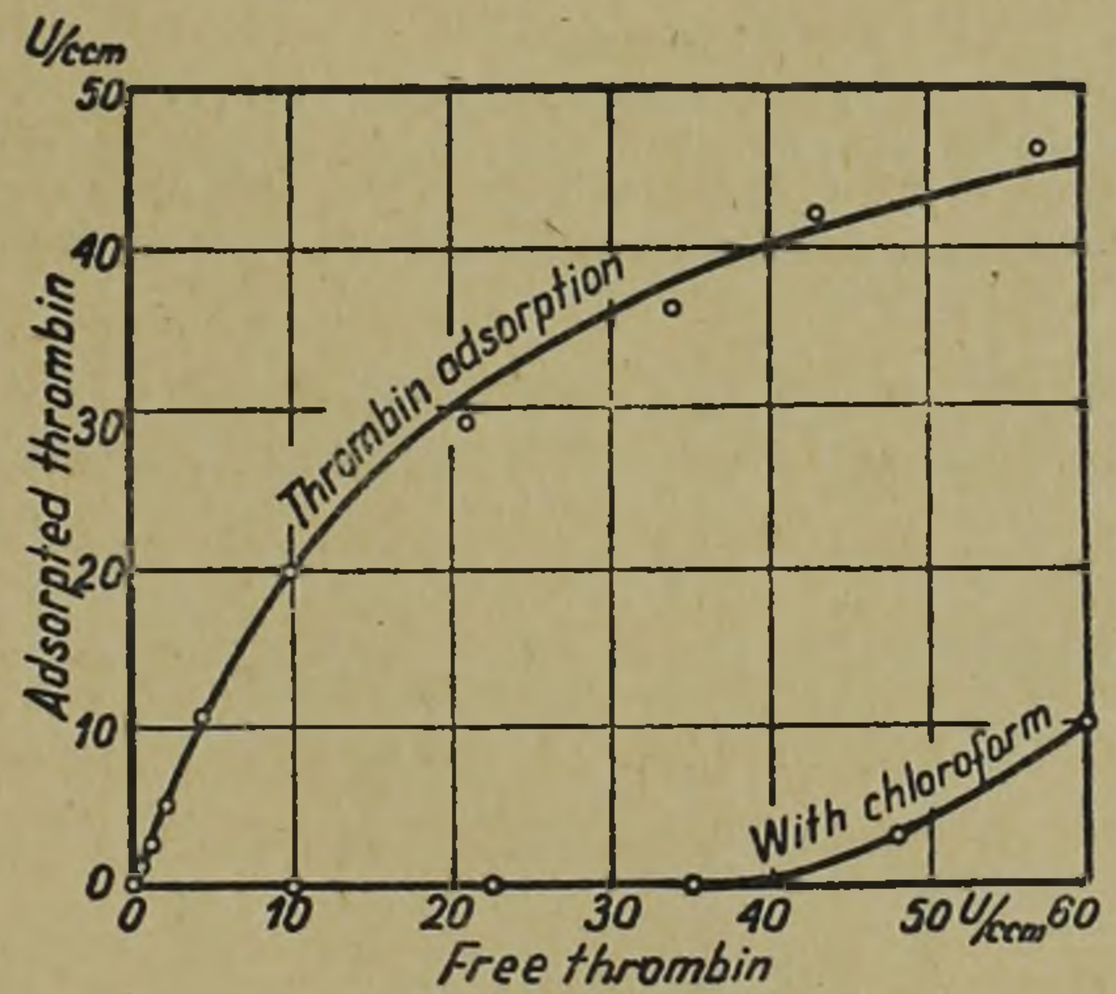

Figure 8.

The curve in Figure 8. demonstrates the results. Table III. shows the data coerrespond with the values calculated on the basis of the Langmuir formula.

$$a = a\infty \cdot \frac{c}{c + b'}$$

a = amount of thrombin adsorbed.
a_∞ = end value of the quantity adsorbed = 60 U/ml.
b' = constant of adsorption = 20.
c = balanced concentration of thrombin.

TABLE III.

Observed and calculated data on thrombin adsorption.

c thrombin u/ml	a observed u/ml	a calculated u/ml
54	46	44
43	42	41
34	36	38
21	29	31
10	20	20
4,3	10,7	10,6
2,1	5,4	5,7
1,05	2,95	3,0
0,44	1,56	1,3

To decide whether thrombin adsorption in the serum is reversible or irreversible — that is, whether by cooling the thrombin is again eluated — I made the following experiment:

To serum kept 10 minutes at 80 C° I added 15 ml macerated 6,5 U/mg thrombin (= about 100 U) in 0,1 ml distilled water, mixed it, then taking samples from the mixture, examined their activity in comparison with that of the previous ones, already known.

0,1 ml fibrinogen
0,1 „ sample of juice ..... 8.

From the clotting time obtained the amount of free thrombin present in the serum was calculated in % of the added thrombin, and finally, diluting the serum mixed with the thrombin in proportion of 1 : 1, 1 : 2, 1 : 4, the changes in the amount of free thrombin due to dilution were established.

The data thus obtained (Table IV.) prove that by dilution the thrombin can be liberated from the adsorption in a reversible way.

TABLE IV.

Changes in free thrombin as a result of dilution.

Dilution	Free thrombin %
0	53
1 : 1,5	69
1 : 2	74,5
1 : 4	79,5

The thrombin adsorbed by the serum is also liberated in a weak alkaline medium. At $pH = 7{,}5$ adsorption of the thrombin significantly diminishes.

INHIBITION OF THROMBIN ADSORPTION BY CHLOROFORM.

According to Minot,[13] Bordet[14] and Stuber, Focke and Chien Shen[15] cloroform accelerate the clotting of blood, and sera whose clotting power is lost can be reactivated by chloroform.[16] According to Minot, chloroform develops its effect by destroying the anti-thrombin.

To investigate this question I determined the accelerating effect of chloroform on clotting. Adding thrombin solution of a strength of 1 U/ml to oxalate plasma, with and without chloroform, I obtained the following clotting times:

0,1 ml oxalate plasma	0,1 ml oxalate plasma
0,1 „ 0,7% NaCl solution	0,09 „ 0,7% NaCl solution
0,1 „ thrombin solution ..... 9.	0,01 „ chloroform
	= 0,1 „ thrombin solution ..... 10.
Clotting time: 58"	Clotting time: 14"

Thus the presence of chloroform really reduces the clotting time markedly. Searching further for the cause of this effect, I tested the inactivating potency of native serum thrombin, with and without chloroform.

1,0 ml 10-times diluted serum	1,0 ml 10-times diluted serum
0,1 „ 100 U/ml thrombin ..... 11.	0,1 „ chloroform
	0,1 „ 100 U/ml thrombin ..... 12.

Samples taken from the mixture were examined in the usual comparisons.

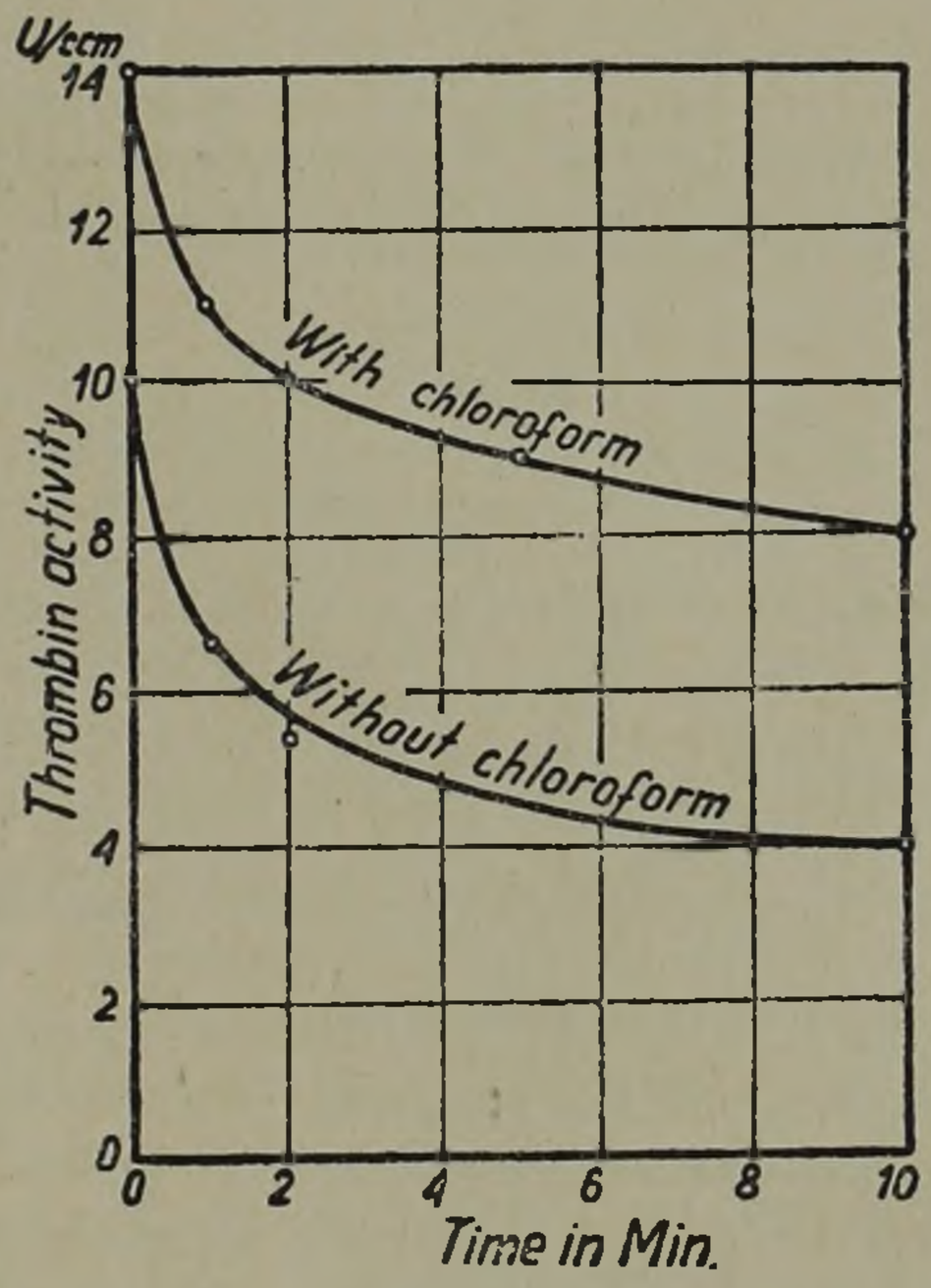

Figure 9.

The curves (Figure 9) obtained on the basis of clotting time clearly show that with chloroform the inactivating effect still functions, but from the parallel rise of the curve we can suppose the cessation of adsorption.

The adsorption-inhibiting effect of chloroform is directly demonstrable in heated serum, where the effect of the inactivator is no longer apparent. Thereafter, as experiments 5,6, and 7 show, I determined the serum's thrombin content with and without chloroform. The results are shown in the lower curve of Figure 8. According to this, on the curve taken with the chloroform, the adsorption is almost entirely lacking, hence the chloroform really does inhibit binding of thrombin by adsorption. The use of bromoform and iodoform give similar results.

THE TEMPERATURE COEFFICIENT OF THROMBIN ADSORPTION.

According to the laws of chemical mechanics, the amount of adsorbed substances diminishes with a rise in temperature, desorption follows; that is, the temperature coefficient is negative. Since, according to literary data on thrombin adsorption already cited, the binding capacitly of serum thrombin increases with a rise in temperature. I investigated the problem by using serum previously heated to 80 C° and determined the adsorption at 10° and 40 C°.

at 10°

1 ml distilled water — 1 ml serum
0,1 „ thrombin solution ..... 13. — 0,1 „ thrombin solution ..... 14.

After mixing, the activity was studied in the way already described. Determination of the activity was made at 10 C°. The same experiment, in the same way, was carried out at 40 C°, with the determination made at 40 C°.

TABLE V.

Change of adsorption due to temperature.

	at 10 C°		at 40 C°	
	Clotting time	Activation	Clotting	Activation
Thrombin and serum ...	140"	0,21 U/ml	39"	2,3 U/ml
Thrombin and dist. water	84"	0,57 „	25"	5,2 „
Free thrombin	36,8%		44,2%	

Thus the amount of free thrombin increases at a temperature of 40 C° that is, on increase of the temperature desorption really follows, in conformance to the laws of adsorption. We conclude from the contradictory results of the literary data that the investigators did not distinguish adsorption and inactivating effect from one another and thus the

inactivator-effect — the speed of which increases with increase of temperature (See: the temperature coefficient of the inactivator effect) — concealed the real adsorption.

FERMENTATIVE INACTIVATION OF THROMBIN (INACTIVATING EFFECT).

According to my experiments, the inactivating effects exercised of complete blood, plasma and serum on thrombin differ from one another only within experimental limits of error. This proves that the inactivator does not disappear either during centrifugation nor during the clotting process, nor does the inactivation of thrombin which takes place in clotting essentially diminish its effect.

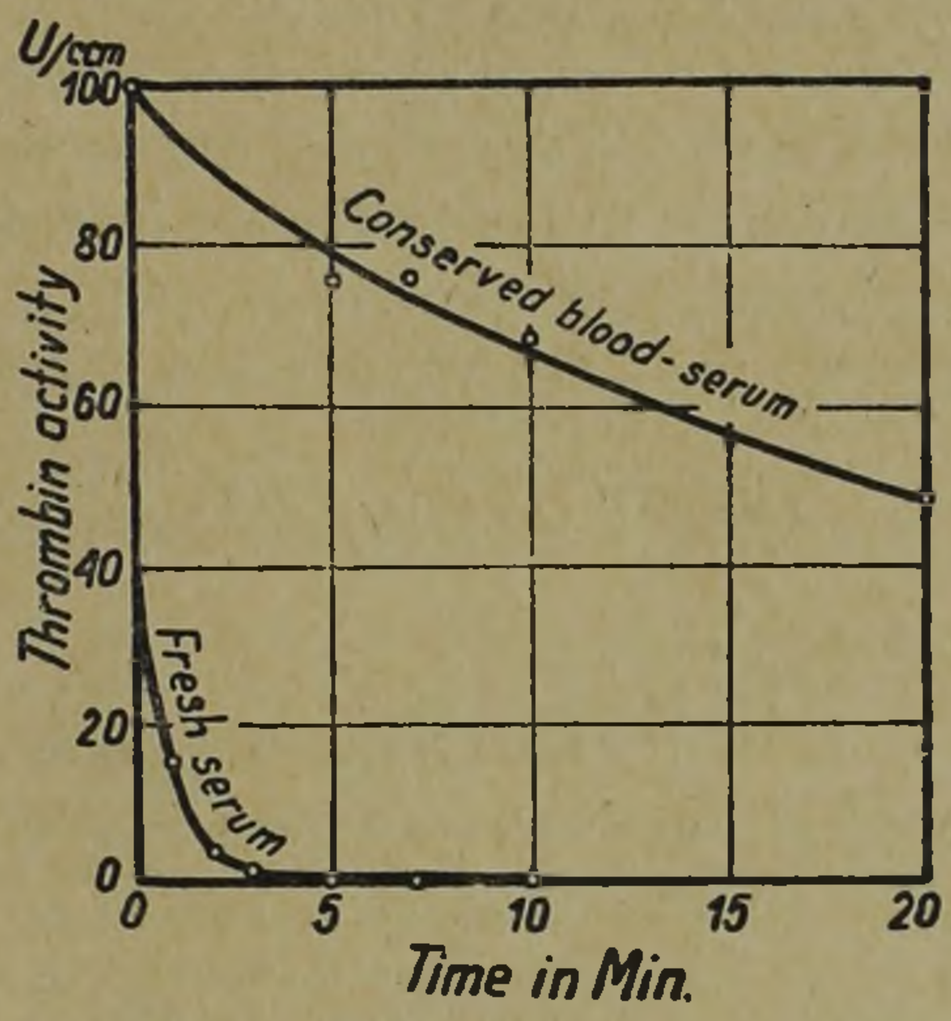

Figure 10.

The curves of the inactivation shown in Figure 4 also prove the strength of the inactivating system, as the inactivation of the thrombin in a quantity of 100—100 U/ml thrombin added one after the other in both cases already falls to about a value of 1 U/ml in blood can inactivate such a great amount of thrombin in the course of a few minutes.

The inactivator is present in human blood serum after 10 months in sterile conditions at room temperature, the same is the case with sera preserved by drying for transfusion,[16] though the thrombin-inactivating potency of these sera is very much less than that of fresh sera (Figure 10). This circumstance warns againt transfusions of preserved sera.[17]

The following observations prove the fermentative character of the inactivating effect.

INFLUENCE OF METAL IONS ON INACTIVATION.

The point of departure in investigating the inactivation of thrombin solutions was the observation that metallic iron increased the inactivation of thrombin. On the basis of this finding I investigated the influence of 0,7% *NaCl*, m/1000 $SnCl_2$, m/1000 $CuCl_2$, m/1000 $AlCl_3$ and m/1000 $FeCl_3$ on the rate of inactivation. In the order described *Na* had the weakest, *Fe* the strongest inactivating effect. The data obtained are a guide to further investigation of the problem, and point out the necessity of using wood or glass vessels and of avoiding metal instruments in the course of purification of thrombin.

THE INACTIVATION-INHIBITING EFFECT OF METAL REAGENTS.

In view of the catalytic effect of metals on thrombin inactivation, experiments were indicated to establish whether metal reagents binding the metals in the form of weakly dissociating compounds have an inhibiting effect on the inactivator. In the first place investigation of chinosol (8-oxychinolin-potassiumsulphate) proved interesting, inasmuch as its ion-complex forming the durability of thrombin it proved a good stabilizing agent.

For the investigations I used the following experimental combination:

1 ml serum
0,1 „ chinosol solution
0,1 „ 100 U/ml thrombin solution ..... 15.

I examined the activation of samples taken from the mixture, in the usual way. The experiment was carried out with chinosol concentrations of $2°/_{00}$, 1% and 2%. The $2°/_{00}$ chinosol concentration curbed the inactivating effect, but complete inhibition was not obtained by the 1% or even the 2% chinosol. In greater concentrations the chinosol inhibits clotting and therefore cannot be used as a stabilizer. The fact that for the stabilizing of purified thrombin solutions the $1°/_{00}$ chinosol concentration proves the most effective follows from the fact that in purified solutions the concentration of the inactivator decreases significantly in consequence of the purification.

Investigating the thrombin inactivating potency of pro-thrombin solutions, we already see from the foregoing (Figure 5), that in comparison with plasma or serum the inactivation is weakened to a great extent. It is thus comprehensible that, adding chinosol to prothrombin solutions, even $1^{00}/_{0}$ solution will almost completely stop the inactivator effect. With 1% chinosol concentrations a complete inhibition is achieved within the investigation period (30 minutes) but, continuing the investigations over a longer time, a quite slow inactivation succeeds this (Figure 11). Naturally the decrease in activity as consequence of adsorption is to be seen here too. Other metal reagents similar to chinosol also produced a trhombin-inactivating effect.

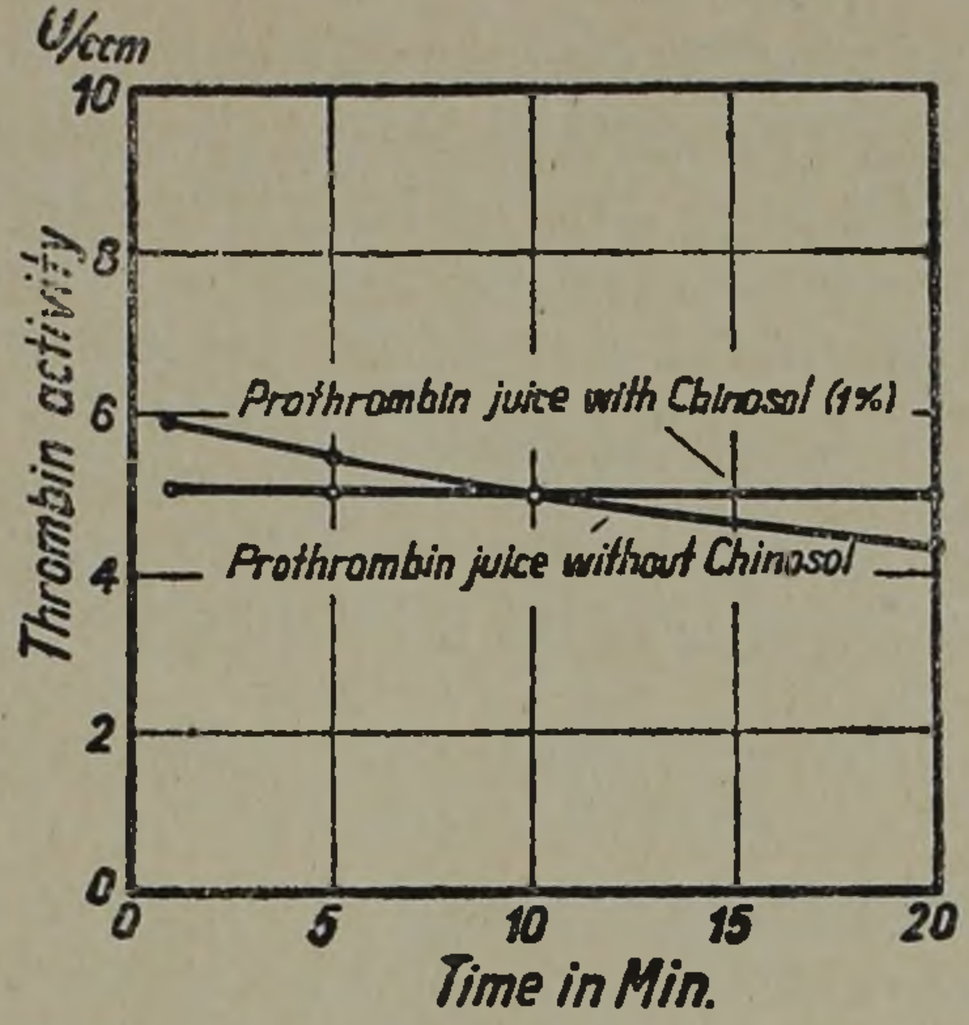

Figure 11.

THE THROMBIN-INACTIVATING EFFECT OF DIALYSED PLASMA.

The effect of metals in increasing, and of metal reagents in inhibiting, inactivation throws light on the fermentative character of the substance causing thrombin inactivation. Experiments carried out with dialysed serum support these findings still better.

I investigated serum dialysed 24 hours against distilled water and diluted 10 times with distilled water. The thrombin consumption of dialysed plasma 10-times diluted was observed in the same way (Figure 12).

Between the two curves there is a significant difference. The dialysed plasma has lost a good part of its inactivating capacity. Determining, as a control, the thrombin inactivating capacity of the dialysing

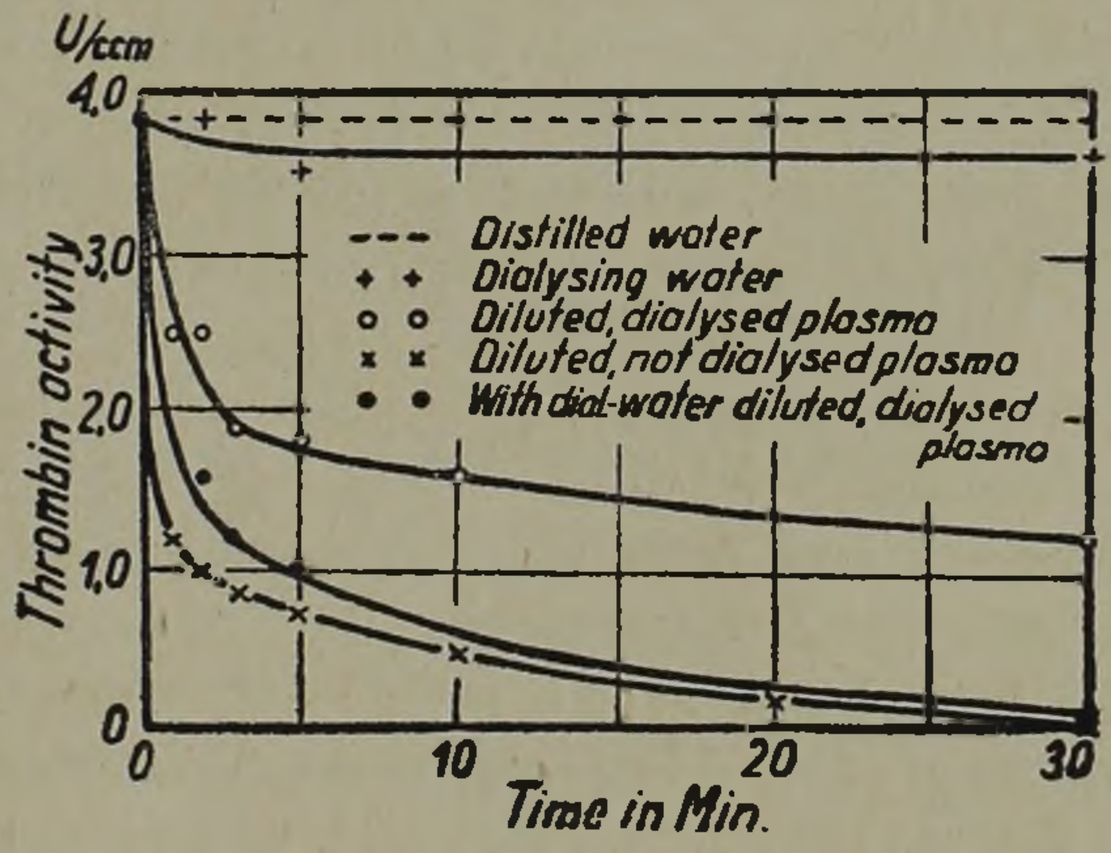

Figure 12.

water, its curve ean together with the line of the distilled water; i. e., it contains no inactivating substance. On the other hand, dialysed plasma diluted with dialysing water gets its inactivating capacity back again, which shows incontestably that in consequence of dialysis some important constituent of the inactivating material has got into the dialysing water. It is an important fact that the dialysed plasma also gets its thrombin inactivating capacity back if, in place of the dialysing water, a diluted solution of some iron salts is added to it (e. g. $FeCl_3$). This again is evidence of the fermentative character of the inactivating material.

CHANGE IN THE INACTIVATION REACTION-SPEED FACTORS DUE TO TEMPERATURE.

The experiment carried out at 26 C° described on page 102, was repeated at 16 and at 36 C° and with the aid of kinetic calculations, the reaction-speed factor at these temperature was established. I calculated the mean of the k-value without regarding the data of the first time interval. The following three k-values were thus obtained.

TABLE VI.

Change in reaction-speed factor due to temperature.

Temperature	Reaction-speed factor *(k)*
16 C°	0,27
26 C°	0,47
36 C°	0,78

According to these data, the rise of the reaction-speed factor for 10 C° is between 10—26 C° (0,47/0,27) = 1,74 times, between 26—36 C° (0,78/0,47) = 1,66, hence the average increase is 1,7 for 10 C°. This corresponds to the thermodynamically proved observation that the value of the reaction-speed factor is generally doubled by an increase of 10 C°.

TEMPERATURE OPTIMUM OF THE INACTIVATOR.

It can be established from the experiments previously described that at approxinately 70 C° the thrombin inactivator loses its inactivating capacity. Thus it was to be expected that the k-values reaching a maximum with a rise in temperature would again diminish. To establish the temperature optimum for the inactivator effect, I carried out the following experiment:

I determined the values of the reaction-speed factor in 10 intervals between 15 and 55 C°; that is, at 5 C° intervals. At higher temperatures (above 40 C°) the activity of thrombin solutions diminishes even without inactivator. This circumstance was taken into consideration in calculating the k-values and correction was made. The k-values obtained from this experiment are given in Figure 13.

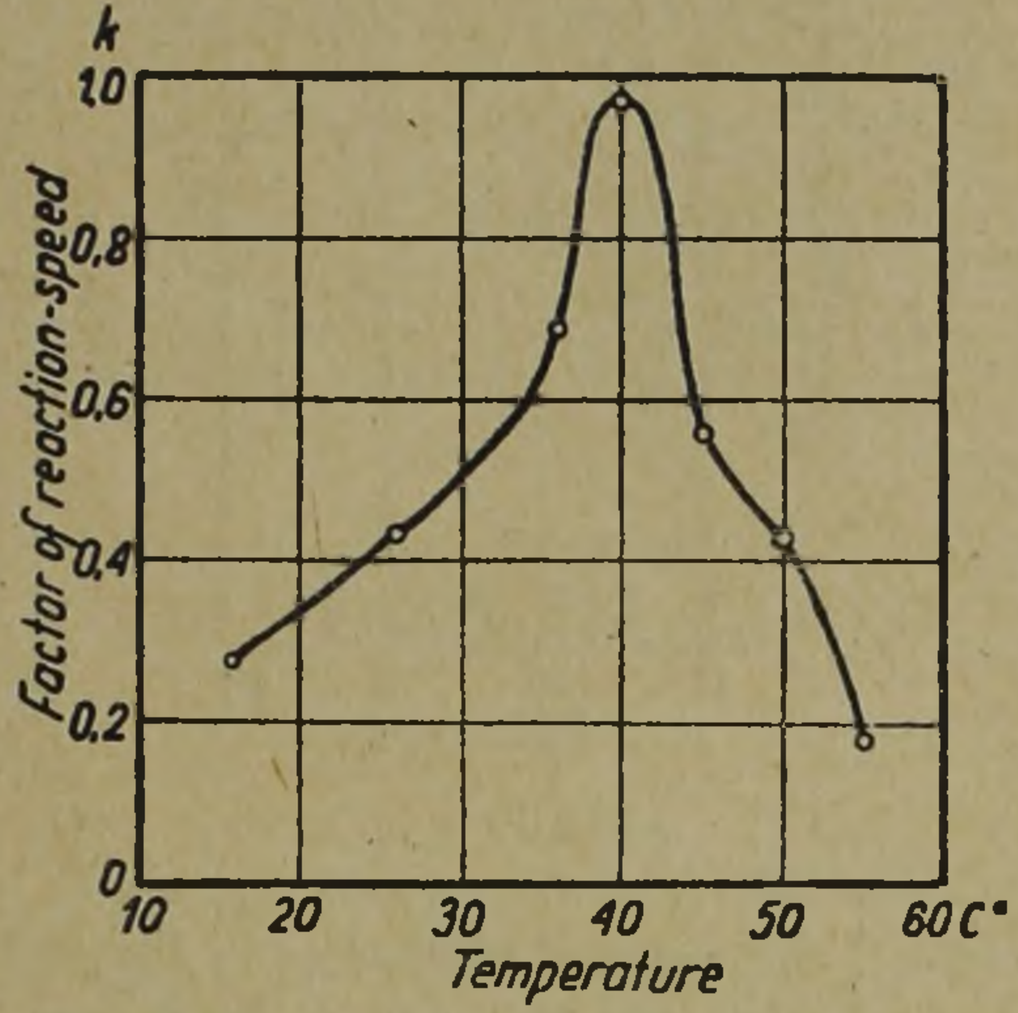

Figure 13.

According to the curve, the optimum of thrombin-inactivation is very sharply at 40 C°, hence in reality it is also adjusted to body-temperature. From the Figure, moreover, it is also clear that the speed-factor value of 1,7 for 10 C° is valid only up to 40 C°.

ESTABLISHMENT OF THE ACTUAL THROMBIN CONTENT OF PLASMA.

At the beginning of this communication I referred briefly to the fact that in native blood the thrombin activity in clotting does not give a true picture of the amount of thrombin actually formed by the blood, as a great part of the thrombin created is inactivated. In investigating the inactivation I established that the disappearance of the thrombin is the result of a sudden adsorption and a fermentative inactivation, and secondly, found a successful method for inhibiting both the adsorption and the fermentative inactivation, therefore using these processes, the amount of thrombin actually produced in the plasma could be established.

Diluting 1 ml oxalate plasma 15 times and acidifying with acetic acid, I separated the pro-thrombin precipitate formed. After solution of the precipitate in a Ca-free Ringer solution, I impregnated it with chloroform and added 1 $/_{00}$ chinosol then recalcified the solution. 1 ml of a 1 : 300 dilution of the thrombin solution formed, was added to 1 ml fibrinogen, produced clotted in 1 minute; i. e., the activity was 300 U/ml (Figure 14). The activity of the solution obtained remained unchanged for hours.

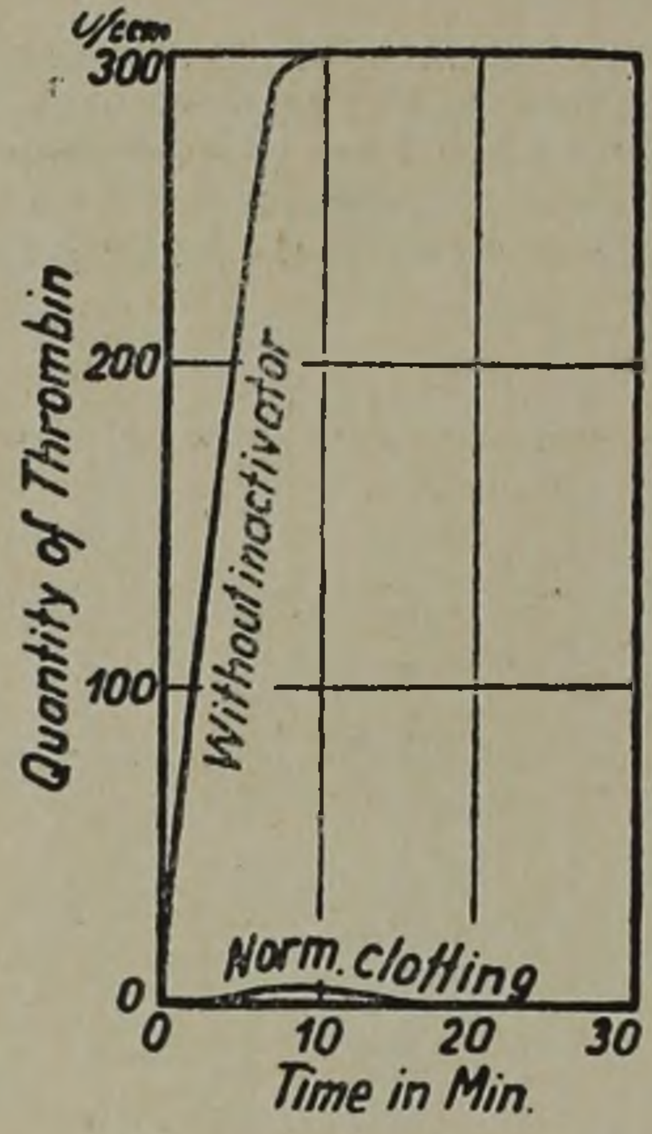

Figure 14.

According to this, the amount of thrombin formed in the presence of chloroform and chinosol is 100 times that which can be demonstrated in normal clotting.[18] The result here obtained serve as a guide, therefore, to the production and conservation of thrombin.

SUMMARY.

The decomposition of thrombin preparation is not of bacterial origine, but is brought about by chemical causes.

The same inactivating system which lead in native blood or in serum to the disappearance of thrombin decreases the potency of purified thrombin solutions. The inactivating substance is present in purified preparations as an impurity.

The process of thrombin inactivation is composed partly of adsorption, partly of fermentative inactivation. The adsorption is reversible and follows the Langmuir adsorption isotherm. Fermentative inactivation is of the monomolecular reaction type. The adsorption is inhibited by chloroform, the fermentative inactivation by metalbinding reagents.

The laws adsorption and inactivation were investigated.

After rendering the inactivating substances ineffective an amount of thrombin about 100 times that which can be demonstrated in normal clotting is found.

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ON THE NATURE OF CROSS-STRIATION.

WITH 13. FIG. IN TEXT.

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INTRODUCTION.

In spite of the great number of theories proposed the cross-striation of muscle has hitherto found no adequate explanation. The Szent-Györgyi groups has shown[1] that the contractile matter is built of two proteins, myosin and actin (Straub)[2] which together form a fibrous complex, actomyosin. The electron microscope has revealed (Hall, Jakus and Schmitt[3] Rózsa and Staudinger[4] that the actomyosin filaments run continously through *A* and *J* segments. These filaments having a positive double refraction *(DR)* it seemed possible that the isotropy of *J* bands is caused by the presence of a negatively *DR* substance, the negative *DR* of which compensates the positive *DR* of the actomyosin filaments.

To decide this question muscle fibres of the rat and other animals were treated in such a way as to dissolve specifically the actomyosin filaments; the remained structure was studied in plain and polarised light.

The experiments have shown that the *J* bands contain a negatively *DR* protein, which we call *N*-protein, which is responsible for the isotropy of *J* bands.

METHODS.

The animals were killed and 1—3 mm wide and 1 cm long stripe were cut out rapidly from the tigh muscle parallel to the fibres. These strips were either immersed simply at once into the ice-cold extraction fluids, or were fixed by acacia thorns on cork plates. For extraction the following fluids were used:

1. Weber's fluid, containing 0,6 m KCl, 0,04 m $NaHCO_3$, 0,01 m Na_2CO_3. To this fluid 1 mg *ATP* (adenosintriphosphate) was added per ml. This fluid is applied usually for the dissolution of the contractile matter. In our experiments it was allowed to act 20 or 60 minutes during which time it was renewed three times and stirred constantly.

2. 0,6 m. *KJ*. After treatment with Weber's fluid the muscle was transferred into this solution and extracted for 20 or 60 minutes. *KJ* depolymerisates and dissolves the fibrous actin. (Straub).

3. Urea-salt solution is Weber's fluid containing 30% urea. The muscle, after extraction with Weber and *KJ* solution was transferred for 20 or 60 minutes into this solution. The urea salt solution dissolves also the *N*-protein.

In several experiments the treatment with *KJ* solution was emitted. All the employed salt solution cause strong swelling. Before microscopic examination this swelling was reverted by placing the muscle into 2, 4 and 8 times diluted solvents and finally into destilled water. Then followed fixation in 10% formaldehyde. The formaldehyde was washed out with water, the muscle dehydrated by alcohol, embedded in paraffn and cut into 10 μ thick slices, which were inclosed in canadabasame dissolved in xylol.

Photogrames were prepared on the Leitz polarisation microscope by a Contax camera. The sign of *DR* was established by the use of Red I gypsum plate which was placed at + 45° to the axis of Nicols. Positive *DR* is revealed by the addition blue, negative *DR* by the subtraction yellow colour.

EXPERIMENTAL PART.

In slices prepared from muscle which was not fixed to cork plates only the alternating dark *A* and light *J* bands could be seen under the microscope. In muscle fixed to cork plates also the *Z* and *M* bans were visible.

Extraction by Weber's fluid. The fluid has a progressive action. Firts the contractile matter in the *A* bands dissolves, whereupon the *DR* of the fibre disappeares. Fig 1 shows a bundle of muscle fibres treated for 20 minutes. At the end, where the solvent acted the strongest the *DR* disappeared and here and there a negative *DR* appeared. Fig. 2 shows the end of the fibres of Fig. 1 at higher magnification. In plain light the cross-striation seemed unchanged.

If the action is more drastical. the dissolution of the contractile matter goes on and the fibres becomes homogeniously negative birefringent. This case is shown in Fig 3, in which Weber's fluid was allowed to act protectively (1 hour) an minced rabbit muscle. Mincing damages the structure and makes the interior of the fibre readily accessible to the fluid.

Weber's fluid + KJ. The action of the Weber + *KJ* is similar to that of the pure Weber's fluid but is more acute because the *KJ* depoly-

merises actin and promotes herewith the dissolution of the contractile matter. In Fig. 4. four zones can be seen, the central untouched zone, a narrow zone, a narrow zone with no *DR*, a wide zone of negativ *DR* and finally a marginal zone in wich all *DR* has disappeared.

The behavior of the single segments of the fibre in plain and polarised light is summed up in Table I. and Fig. 5.

TABLE I.

	A band	*J* band	*Z* line
Untreated muscle.			
Plain light	dark	light	dark
Polarised light	light (anisotrop)	dark (isotrop)	light (anisotrop)
Polarised light with gypsum plate	blue	dark	blue
Treated muscle:			
Plain light	dark	light	dark
Polarized light	dark (isotrop)	light (anisotrop)	dark (isotrop)
Polarised light with gypsum plate	dark	yellow	dark

It is important, at this point to show definitely that it is the former *J* band which becomes negatively *DR* on treatment with Weber or Weber *KJ*. This point is clearly brought out by the subsequent figure. In Fig. 6*a* and *b* an untreated and a treated fibre is shown in polarised light. It shows that it is the former *J* band which becomes negatively *DR* on treatment while the former *A* bands lost its *DR*.

Weber + KJ + Urea. Banga and Szent-Györgyi[5] have shown that strong urea in presence of salts, dissolves the fibrous structural proteins. They also showed that muscle contains a negatively *DR* structural protein which is dissolved by urea-salt solution. One of us (Gerendás) was involved in these experiments. If the *N*-substance is a protein and is identical with the structural protein described in muscle by Banga and Szent-Györgyi, we can expect that urea salt solution will make the negativ *DR* disappear. The experiment shows that on treatment of the fibre, extracted for 20 minutes Weber, that for 20 minutes with *KJ*, the cross striation and *DR* disappeares under action of urea. Fig. 7*a, b*. This shows that urea dissolves the *N*-substance responsible for the negative *DR* of the Weber + *KJ* extracted fibres.

If the intact muscle is extracted directly with Weber containing urea, varied pictures are obtained with all transitory forms described above. A transitory form between the positive and negative birefringent

parts inside one fibre is given in Fig. 8. In insect muscle (Hydrophylus) owing to the great diameter of bands can be followed very distinctly and Fig. 9 is made from such muscle. These figures 8 and 9 also show distinctly that it is the *J* band which acquires negative *DR*.

In some cases fibres were obtained by Weber + Urea extraction, in which also the negative *DR* disappeared but in plain light some sort of cross striation remained. Fig. 10*a* and 10*b*.

If the muscle is extracted with Weber's solution, then *KJ* and finally with urea, this latter solution acquires a negative birefringence of flow.

The question may arise whether the negatively *DR* substance responsible for the isotropy of *J* bands and appearently identical with the Banga and Szent-Györgyi's structure protein is not formed from actin or myosin under influence of our reagents by a rearragement of the micellar structure. The experiments show that both actin and myosin have a positive *DR* and are not converted into a negatively double breaking substance under action of *KJ* or ure salt solution, as shown by their birefringence of flow.

FURTHER OBSERVATION.

The authors think to have shown that the isotropy of *J* bands is due to the compensation of the positive *DR* of the actomyosin filaments by a negative *DR* of the *N*-protein. This they tried to show by extracting specifically the *N*-protein leaving the actomyosin in place. In this case the muscle fibres should assume a uniformous positive *DR* and cross striation should disappear. This can be done by subjecting the muscle to putrefaction whereby the *N*-protein is decomposed and the more resistant actomyosin is retained. This experiment has been done previously by different authors[6, 7]. We employed the thigh muscle of Dytiscus marginalis for this experiment. The result is illustrated by the Fig. 11.

If our results are correct we have to expect that the numeric value of the negative *DR* of the *J* bands, after elimination of actomyosin, is identical with that of the *A* bands before extraction, the sing being opposite. In order to decide this question the *DR* of muscle fibres was measured by means of the Babinet compensator. The average value, obtained on 12 different fibres, treated with Weber + *KJ* and embedded in xylol-canada-balsame $n_\gamma - n_\alpha = -39 \times 10^{-4}$. Identical positive *DR* was found in untreated fibres: $n_\gamma - n_\alpha = 40 \times 10^{-4}$. Naturally, the *DR* measured depends on the refractory index of the embedding medium. Experiments on this line will be discussed in detail later. The nature of the *N*-protein, which seems to be a nucleoprotein, will also be delt with at a later occasion.

SUMMARY.

Pieces of rat muscle were treated with different solvents and the effect observed under the microscope.

In fibres, treated with Weber's alcaline salt-solution, the double refraction of *A* segments was found diminished or completely missing while negative double breaking appeared. In plain light no change was observed.

If this treatment was followed by extraction with *KJ* solution the *J* bands became negatively double-refracting. In plain light no alteration of the structure was observed.

If the *KJ*-treatment was followed by extraction with strong urea, both the double refraction and the microscopic structure disappear.

It was deduced from these experiments that the isotropy of *J* bands can be attributed to the presence of a negatively double-refractant protein which has a periodic distribution and compensates the positive double refraction within the *J* segments. This substance was called *N*-protein.

Our thanks are due to Mrs. Matoltsy for her helpful cooperation during part of this work.

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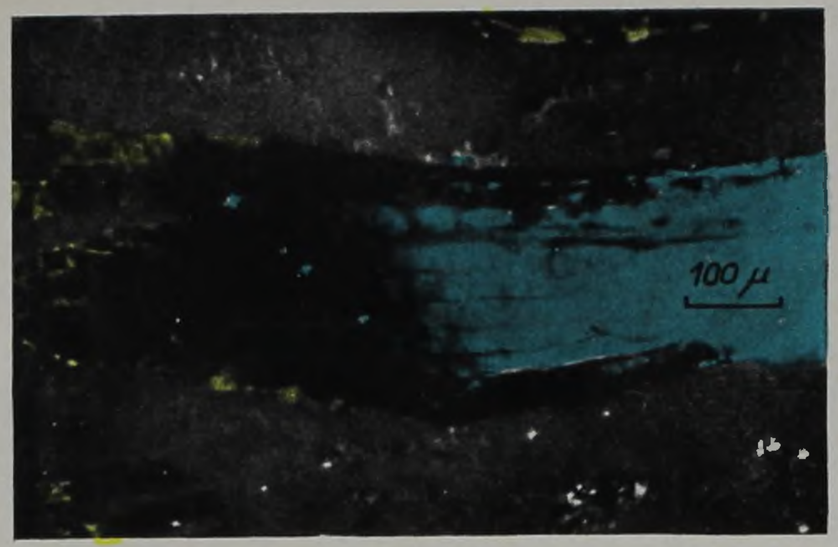

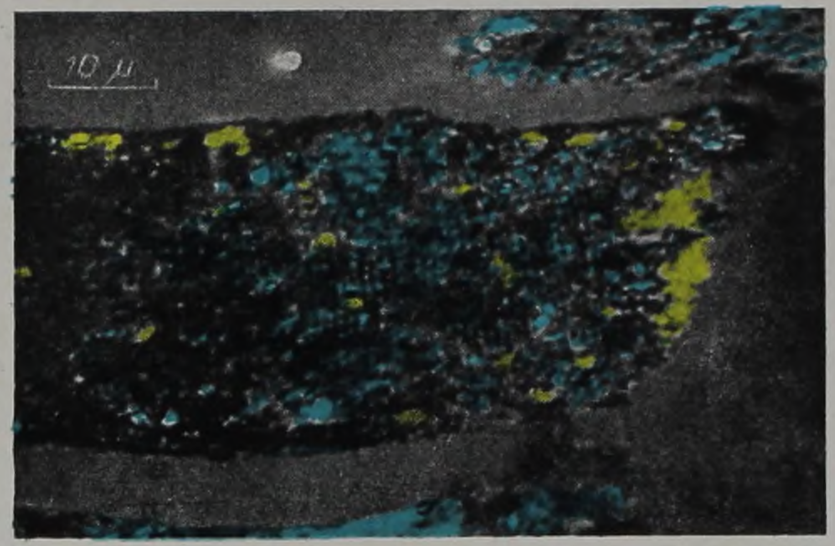

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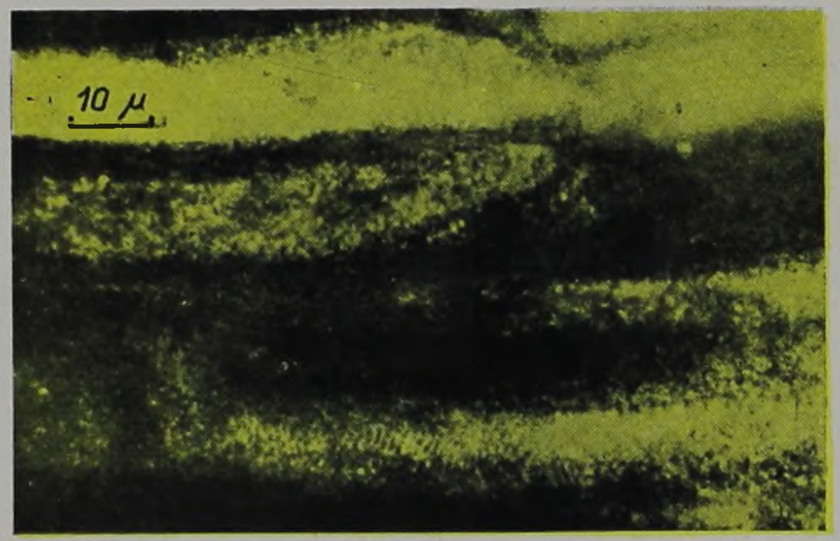
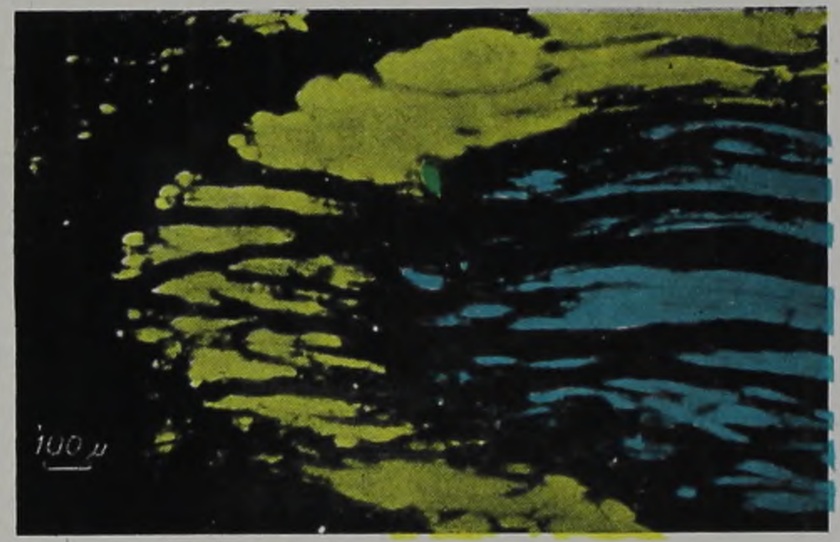
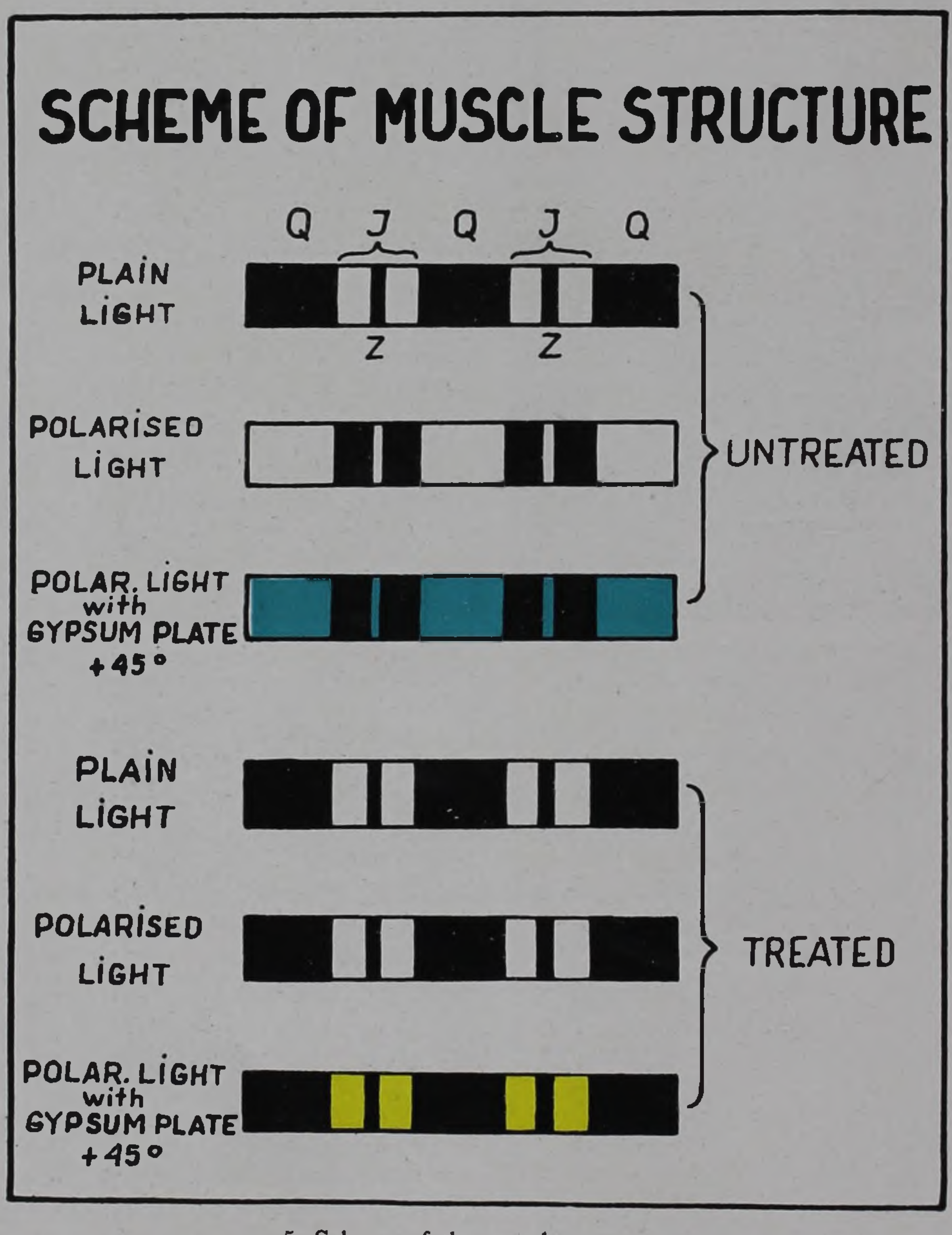




4.

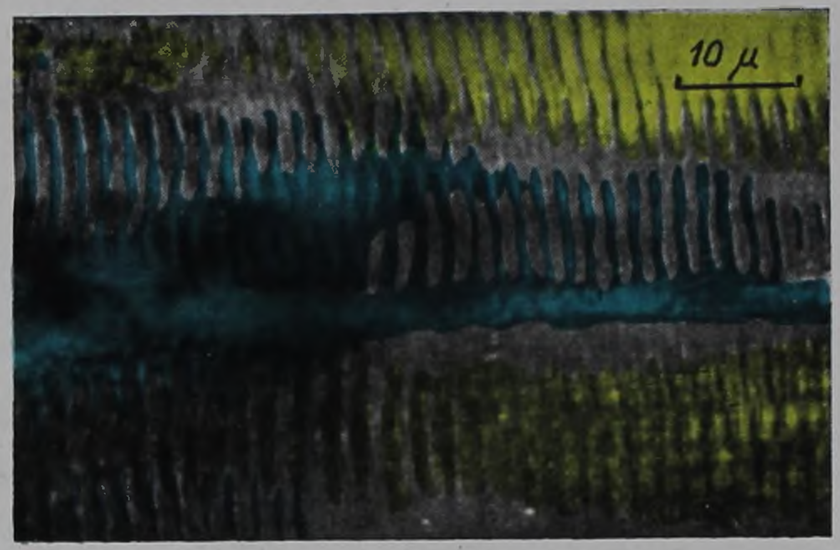
1. Fiber bundle with Weber solution (polarised light, gypsum plate). At the end of the fibres is „—" DR. 2. Fiber treated with Weber solution (polarised light, gypsum plate). The „—" double refraction is visible on some parts of the fibre. 3. Rabbit muscle strongly extracted with Weber solution (polarised light, gypsum plate). The "—" double refraction dominates. 4. Fibre bundle treated with Weber + KJ solution (polarised light, gypsum plate). On the border of the fiber bundle penetrated by the solvent the fibres are negative double refractant.



5. Scheme of the muscle structure.

6/b.

6/a.



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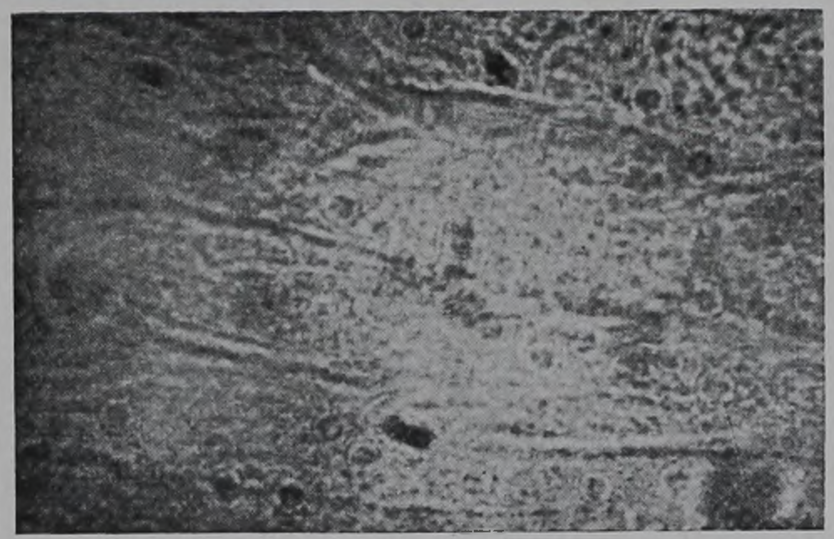
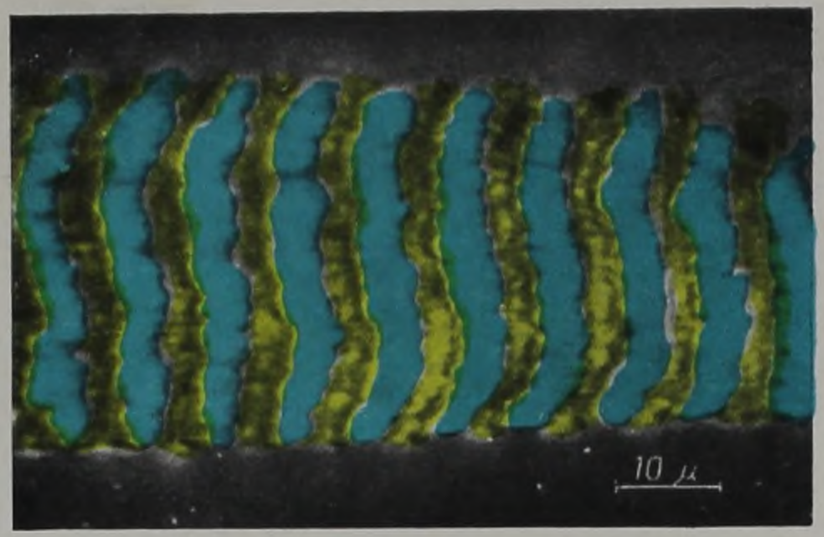


7/a.

7/b.

6/a. Untreated muscle (plarised light, gypsum plate). A and Z bands are blue, "+" double refractant, J band is dark (isotrop). 6/b. Muscle treated with Weber + KJ (using high enlargement, polarised light, gypsum plate). Where the contractile proteins are dissolved the A and Z bands are dark (isotrop), while the J band, in which the negative double refractant material is retained, is "—" double refractant. 7/a. Fibers treated with Weber + KJ + Urea-salt solution (plain light). The structure of the fibres is desorganisated. 7/b. The same fibers 7a in polarised light. No DR. 8. Muscle fiber treated with urea-salt solution (polarised light, gypsum plate).Transition of the "+" into "—" double refraction in a single fiber.

9.



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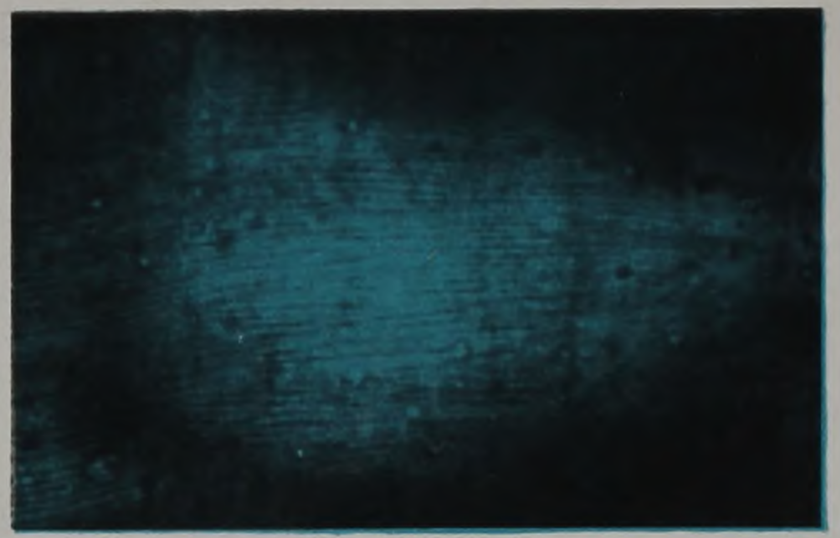
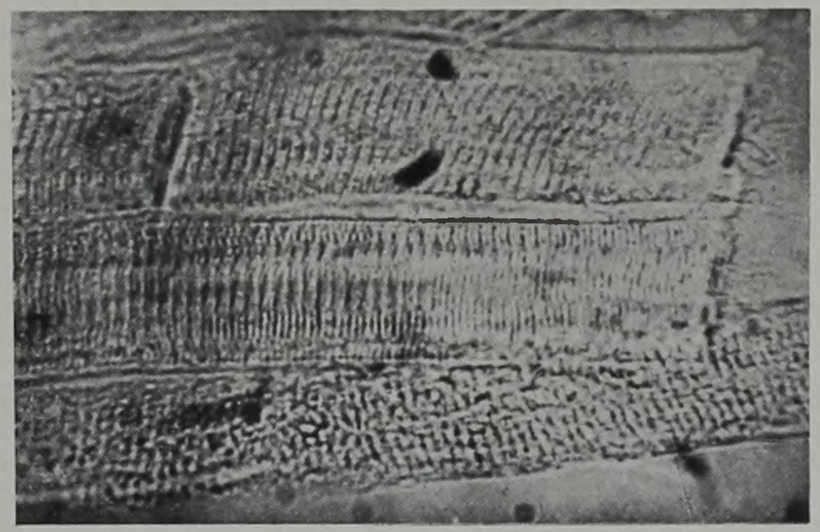
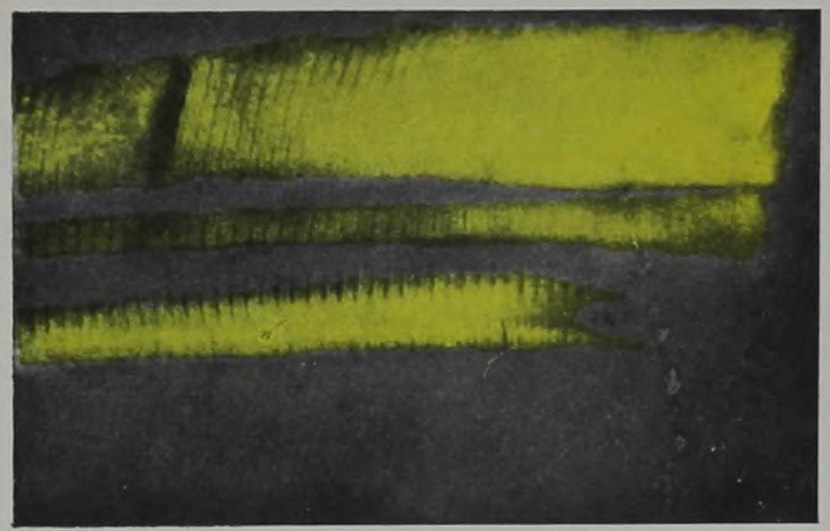


10/a.





10/b.

120d

9. Muscle fiber of the leg of Hydrophilus treated with Weber-urea solution polarised light, gypsum plate). In the A bands the contractile proteins still show "+" double refraction; the J bands are negatively double refracting. 10/a. Fibers treated with urea-salt solution (plain light). In all the three fibers a cross-structure can be seen. 10/b. The same fibers as in Figure 10a (polarised light, gypsum plate). The upper fiber is "—" double refracting. In the middle fiber here and there the "—" double refraction disappears. In the lower fiber, where the "—" double refracting material is dissolved, no double refraction is to be seen. 11. Dytiscus leg muscle exposed to putrefaction (polarised light, gypsum plate). The myofibrils are in their whole lenght "+" double refracting, owing to the decomposition of the negative double refracting material.

SPECTROSCOPIC INVESTIGATION OF THE N-PROTEIN IN MUSCLE.

WITH 1. FIG. IN TEXT.

BY M. GERENDÁS, P. SZARVAS AND A. G. MATOLTSY.

FROM THE HUNGARIAN BIOLOGICAL RESEARCH INSTITUTE, TIHANY, AN THE GENERAL CHEMISTRY INSTITUTE OF THE UNIVERSITY OF BUDAPEST.

(RECEIVED FOR PUBLICATION 30. 8. 1947.)

INTRODUCTION.

An explanation of the isotropy of the *J*-band — as compensation isotropy — was obtained by a demonstration of the negative double refracting *N*-protein in striated muscle.[1] It could be shown from the *N*-material.[2] — on the basis of its solubility in urea and its negative double refraction — that it is the same as the negative double refraction of flow structure protein[3] isolated from the muscle by Banga and Szent-Györgyi. As Banga and Szent-Györgyi, because of the high phosphorous content of the material they isolated, supposed it to be a nucleoprotein, it was natural to determine its ultra violet absorption spectrum, to establish whether our material would give absorption lines characteristic of a nuclein basis.

METHOD.

We extracted the positive double refracting contractile proteins, myosin and actin, from chopped rabbit muscle, using Weber solution (0,6 mol KCl + 0,04 mol $NaHCO_3$ + 0,01 mol Na_2CO_3) and KJ solution (0,6 mol KJ). After dissolution we prepared some fibres histologically and established that the *Q*-stripes lost their positive double refraction, while the *J*-bands became negative double refracting. Then, following the methods of Banga and Szent-Györgyi, we treated the fibres with a Weber + 30% urea solution and controlled by polarized optical examination that the fibres also lost their negative double refraction, while on the contrary the solution gave a negative double refraction of flow; hence the *N*-material went into the solution. We determined the ultra violet adsorption spectrum of the solution thus obtained with a Hilger E 3 quartz spectrograph and a Judd-Lewis sector-photometer. The protein content of the solution examined was 1 mg/ml. We used Weber-

urea solutions for comparisons. We calculated the extinction-coefficient value on the basis of the formula

$$\varepsilon_{1\,cm}^{1\,\%} = \frac{1}{c \cdot d} \cdot log \frac{J_\bullet}{J}$$

In a similar way we also determined the spectrum of actomyosin solution prepared from the crystallised myosin prepared by Szent-Györgyi's process[4] and from purified actin prepared by Straub's method.[5] The solution contained 1 mg/ml protein (0,834 mg myosin + 0,166 mg actin) in 0,5 mol *KCl*. The solution used for comparison was 0,5 mol *KCl*.

EXPERIMENTAL RESULTS.

In the spectrum of the material extracted with urea (Figure 1, curve *a*), we obtained a maximum at 2650 *Å* characteristic of nuclein basis adsorption and at 2800 *Å* a protruberance characteristic of the presence of protein, so that also on the basis of the *N*-material's spectrum it can be considered a nucleoprotein. The curve corresponds well with the spectrum of the crystallised tobaccco mosaic virus-protein[6],[7] and with Caspersson's[8] adsorption spectrum found in the *J*-bands

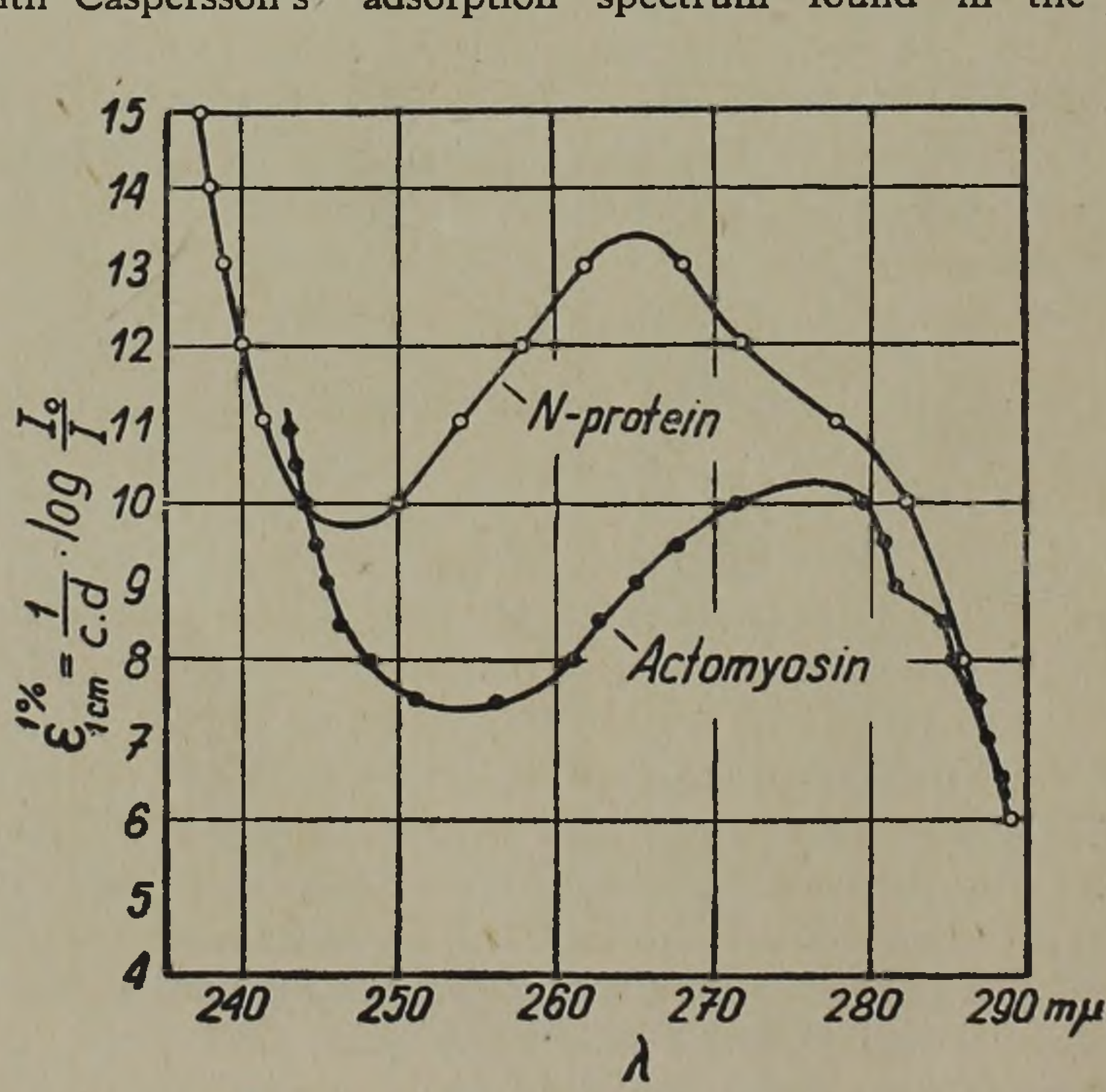


Figure 1.

of striated muscle. The spectrum of the actomyosin solution determined by us is, however, a simple protein spectrum whose maximum is about 2760 *Å* and is similar to the curve obtained in the *A*-band of muscle by Caspersson. This finding is in complete harmony with Caspersson's theory that it is the proteins in the *A*-band, in the *J*-band principally the nuclein-bases, which absorb. Caspersson, however, considers the nucleotid spectroscopically shown in the *J*-band to be adenyl acid and its quantity in the muscle — on the basis of approximate calculations — to be only 0,1—0,2%.

Our experiments show that the line of adsorption at 2650 *Å* of material extracted with urea could not originate from adenyl acid or other soluble nucleotid, because this was certainly eliminated from the muscle during the treatment with Weber solution. The fact that the *N*-material only dissolves under the effect of a Weber solution containing 30% urea means just that, aside from the soluble adenyl acid system, the muscle contains a great quantity of bound nucleotid in the *J*-band.

At present we do not know to what type of protein to connect these nucleotids, therefore we cannot carry out determinations of the amount of the nucleotids or calculations of the nucleotid-protein proportion. The purin-nitrogen[9] and pentose[10] content of the muscle however, also allow us to consider the *N*-material as a nucleo-protein.

SUMMARY.

We determined the ultra violet adsorption spectrum of actomyosin and *N*-protein extracted from muscle. The *N*-material gives a curve characteristic of a nuclein basis, the actomyosin of proteins. We established that, aside from the adenyl acid system, the muscle contains bound nucleotids.

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DOUBLE REFRACTION OF THE *N*-PROTEIN.

WITH 1. FIG. IN TEXT.

BY M. GERENDÁS AND A. G. MATOLTSY.

FROM THE HUNGARIAN BIOLOGICAL RESEARCH INSTITUTE, TIHANY.)

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INTRODUCTION.

In a foregoing paper we described,[1] in cross-striated muscle, a negatively double-breaking protein which is located in the *J*-segments and compensates by its negative double refraction *(DR)* the positive *DR* of the actomyosin filaments. In this paper we propose to give a more detailed analysis of the *DR* of this protein.

METHOD.

We used rat's muscle for our experiments. The pieces of muscle were put into a Weber solution (0,6 mol KCl + 0,04 mol $NaHCO_3$ + + 0,01 mol Na_2CO_3) for 20 minutes to dissolve the contractile substance of the muscle, and then for 20 minutes into a KJ solution (0,6 mol KJ). The fibers were then reduced to their normal size in solutions of diminishing concentrations, fixed and embedded in paraffine. Slices of 10 μ were prepared from the paraffine blocks and, dissolving the paraffine with xylol, the slices imbibated in the following order:

1. xylol. 2. alcohol 3. water. 4. formaldehyde solution. 5. water. 6. alcohol. 7. benzyl alcohol. 8. alcohol. 9. a mixture of aniline and alcohol. 10. aniline. We transferred from one solution to another through intermediate mixtures.

The imbibition of the preparations took place on the microscopic stage without their being moved from their palaces. In this way we could make sure that we carried out the *DR* determination on the same fibre in the same place.

The *DR* value was calculated[2] on the basis of the formula

$$n_{\parallel} - n_{\perp} = \frac{\Delta\lambda}{d}$$

where $n_{||} - n_{\perp}$ is the total *DR* of the threads, $\Delta\lambda$ the difference between the ways in $m\mu$, measured with a Babinet compensator, and d the thickness of the microscopic slices, also in $m\mu$.

The total *DR* is composed of the formal-*DR* $(n_a - n_e)$ and its own-*DR* $(n_\varepsilon - n_\omega)$ in the following way[3]:

$$(n_{||} + n_{\perp}) = (n_a - n_o) + (n_\varepsilon - n_\omega)$$

We drew the measured total-*DR* values in their function as n_2 index of refraction of the imbibating solutions, and, on the basis of the curves obtained and the foregoing formula, determined the values of its own- and the formal-*DR*.

RESULTS OF THE MEASUREMENTS.

The dissolution of the muscle's positive double refracting material and thereupon the prominence of the negative *DR* material is a continuous phenomenon. In some fibres this process is only just beginning, in others it is already in an advanced stadium. But just this is what is significant in this phenomenon, and from the large number of fibres examined we were able to formulate a series of curves (Figure 1). The curves show a consistent transition from the "+" double refracting, intact fibres, to the "—" double refracting.

Curve *a* in Figure 1. is the imbibition curve of an untreated fibre, which corresponds approximately to similar data in the literature. Curve about $n_2 = 1{,}48$ reaches its minimum. At this point the fibre's formal-*DR* is eliminated, and so the value obtained here is equal to the fibre's own-*DR*.

$$(n_\varepsilon - n_\omega) = +\,40.$$

The decrease or increase in the values of the refractive index (n_2) of the imbibation solution results to an equal degree in raising the curve. This, as interpreted by the Wiener theory, means a positive formal-*DR*.

We found the total-*DR* value of the untreated fibre in water to be + 60, consequently the value of the formal-*DR* referring to water is

$$(n_a - n_o) = (n_{||} - n_{\perp}) - (n_\varepsilon - n_\omega) = (+60) - (+40) = +\,20.$$

The more strongly dissolved the fibres we examined, the lower the imbibition curve we obtained. The imbibition curve of the greatest number of the fibres is like to the *f* curve. Therefore we got a mean-value for the negative *DR* fibres embedded in xylol-Canada balsam of

$$(n_{||} - n_{\perp}) = (n_\varepsilon - n_\omega) = -\,39.$$

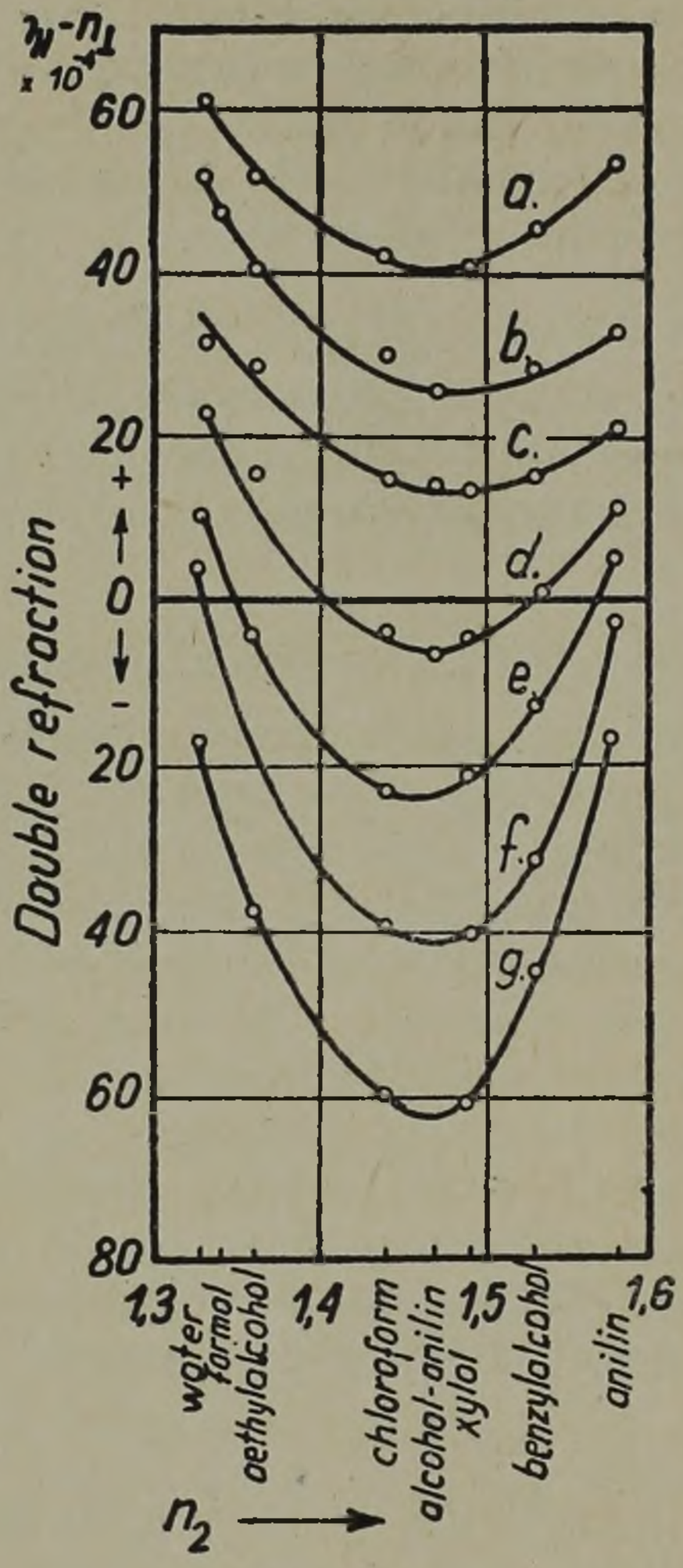

Figure 1.

From the series of curves it is also apparent that even when the index of refraction of the imbibition solution diminishes — as for example putting it into water — the total-*DR* will be positive, or at least in the neighborhood of a 0 value. This explains why the appearance of the negative *DR* cannot be noticed in the course of dissolution when Weber solution is used, because its index of refraction $(n_2 = 1{,}339)$ scarcely differs from that of water. Besides such index of refraction the imbibition curves in general still fall to a positive area and only exceptionally can a negative *DR* fibre be found among them. If we want to observe "—" *DR* present after the dissolution of the contractile substances, then the fibres must

be embedded, after the necessary pre-treatment, in a material with a higher index of refraction.

In the fibres with the strongest negative *DR* (curve *g*) the value of its own-*DR* −63. The total-*DR* in water of these fibres is −9, consequently the formal-*DR* at this point is

$$(n_a - n_o) = (n_{||} - n_{\perp}) - (n_{\varepsilon} - n_{\omega}) = (-9) - (-62) = +53.$$

In fibres changed to negative *DR*, more exactly in the *J*-bands, in demonstrated negative double refracting material, therefore, its own negative *DR* combines with the formal positive *DR*. From this phenomenon we can also conjecture the structural background. On the basis of the imbibition curve in the *N*-material, we must suppose a rod-like micellar structure, built from lamellar-molecules, which is easily conceivable in the presence of nucleotids[4].

SUMMARY.

We determined the curve of imbibition of the *N*-protein remaining in the muscle after the dissolution of the contractile proteins.

On the basis of the imbibition curve the *N*-material has its own negative and a formal "+" double refraction.

From these observations we can suppose a rod-like micellar structure built of lamellar molecules.

Our thanks are due to Mrs. Matoltsy for the helpful cooperation during part of this work.

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ANALYSIS OF THE STRUCTURE OF THE A AND J BANDS OF STRIATED MUSCLE, ON THE BASIS OF IMBIBITION INVESTIGATIONS.

WITH 2. FIG. IN TEXT.

BY M. GERENDÁS AND A. G. MATOLTSY.

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(RECEIVED FOR PUBLICATION 30. 8. 1947.)

INTRODUCTION.

Many researchers have dealt with the determination of the imbibition curve characteristic of the muscle's double refraction, finding that the form of the curve shows its own positive and a formal positive double refraction (*DR*). As among the anisotrope protein components of the muscle only "myosin" was known, they considered the curve obtained a curve of the imbibition of myosin[1], [2], [3]. On the basis of Wiener's theory, they deduced from the positive formal *DR* a rod-like micellar structure, situated in the longitudinal direction of the fibrils, while from its own positive *DR*, chain molecules extending longitudinally inside the micells. This observation also supported the hypothesis that the "myosin" extracted from the muscle — drawn into threads — gave an imbibition curve similar to that of the muscle fibres.

Recently Szent-Györgyi,[4] and his co-workers, especially F. B. Straub[5],[6] demonstrated from the muscle's contractile material that it consists of two proteins: myosin and actin. These two substances taken together form the fibrous actomyosin. The actomyosin filaments can be shown with electronmicroscope. According to the investigation of Hall, Jakus and Schmitt, these filaments which are responsible for the muscle's positive *DR* run the full length of both the *A* and *J* bands, parallel with the long axis of the myofibrils.[7]

In the negative *DR N*-protein demonstrated by us in the *J*-band we recognised still another structural component.[8] It can be stated from these observations that the imbibition curve of the muscle known so far does not derive from myosin but is a resultant curve created of 2 components, the positive double refracting actomyosin and the negative double refracting *N*-protein. From the imbibition curve of untreated

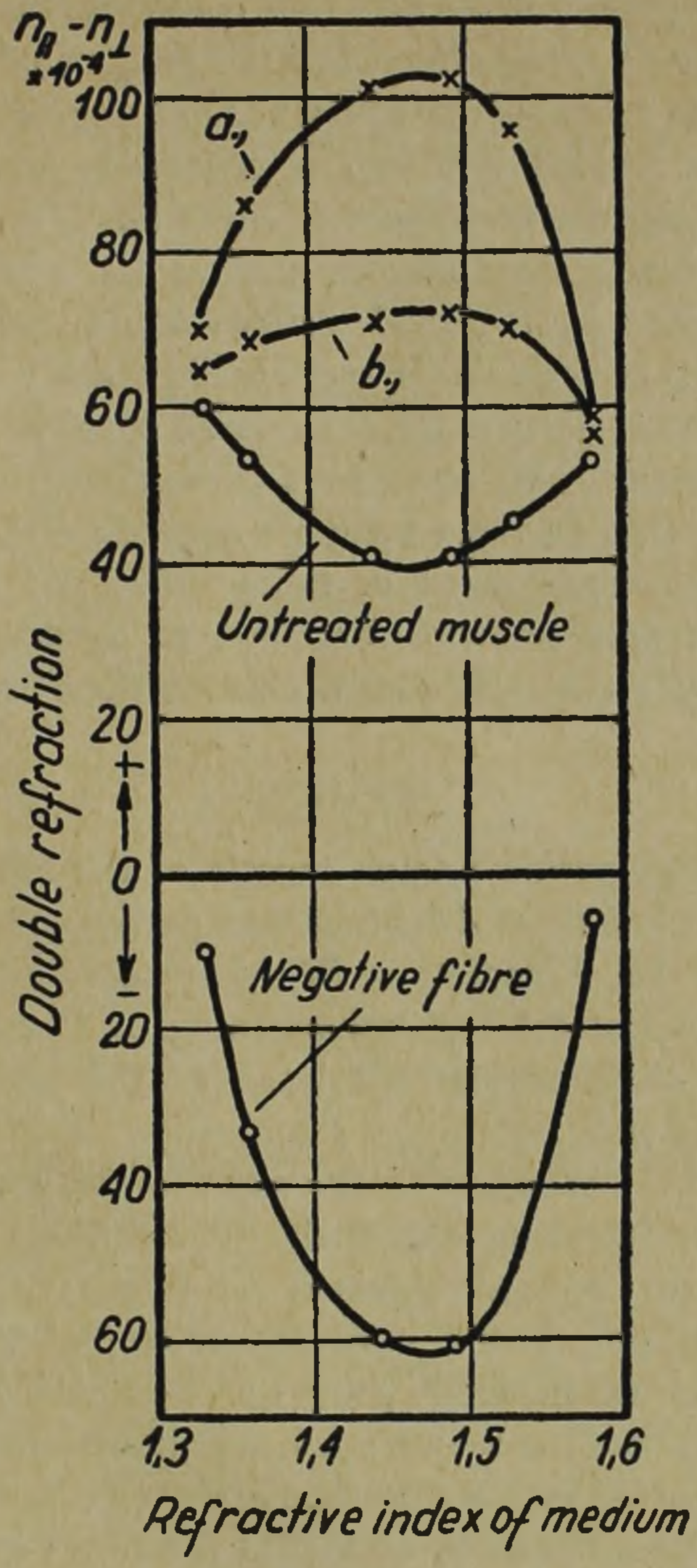

Figure 1.

muscle fibres as resultant curve and from the curve of treated muscle fibres containing *N*-protein, we can calculate the unknown imbibition curve of actomyosin:

$$(n\parallel - n\perp)_{\text{actomyosin}} = (n\parallel - n\perp)_{\text{whole muscle}} - (n\parallel - n\perp)_{\text{negative fibre.}}$$

From our previous communication[9] we took the curves (untreated muscle and negative fibre curve) used for the calculations (Figure 1). The results of these calculations are shown in Figure 1 curve *a*. But our

reckoning is correct only if we take into consideration the data of Banga and Szent-Györgyi[10], to the effect that the amount of the muscle's contractile protein can be twice the amount of the negative double refracting structure protein. Taking this into consideration we get a flatter but still always mounting curve (Figure 1, curve *b*). This curve is the curve of the actomyosin, which has its own positive and a formal negative *DR*. The results obtained by these calculations could also be confirmed by direct experiment, as follows. If our findings are correct, then in the *A* band of the mussle — where the actomyosin could be observed independent of the *N*-protein — we must get a curve giving its own positive and a formal negative *DR*. In the *J* band we must find the imbibition curve resulting from the combination of the positive and negative structure components. The problem was, therefore, to determine separately the imbibition curves of the *A* and *J* bands. This was done in the following way:

METHOD.

We used large periodic thigh muscle of the Hydrophylus for the experiments. The muscle was investigated partly untreated, partly after treatment with Weber-*KJ* solution. We prepared the fibres in the way previously communicated,[9] and imbibated them. For measuring the *DR* we directed the dark interference band of the Babinet compensator longitudinally into the fibre under examination. The line shifted to the left in the *A* and *Z* bands and to the right in the *J* band. In this way it was possible to determine separately the *DR* value in the *A* and the *J* bands. From the picture obtained in media of different indices of refraction we prepared a series of microphotographs and controlled the values determined with the Babinet compensator with them. For these photographs we used a Contax camera provided with a Babinet compensator with a side-tube observer (Phoku). After carrying out the series of measurements, we turned the fibres in a cross-direction, determined in the usual way the imbibition curve of the "whole muscle" at a lower magnification.

EXPERIMENTAL RESULTS AND DISCUSSION.

In Figure 2. we show in the same muscle fibre the imbibition curve of *A* and *J* bands lying side by side and the imbibition curve of the "whole muscle" measured in the same place.

The curve of the *J* band arches downward and its lower part falls into negative territory. In the *J* band therefore its own negative *DR* continues with the formal positive *DR* (Figure 2, *I*-band).

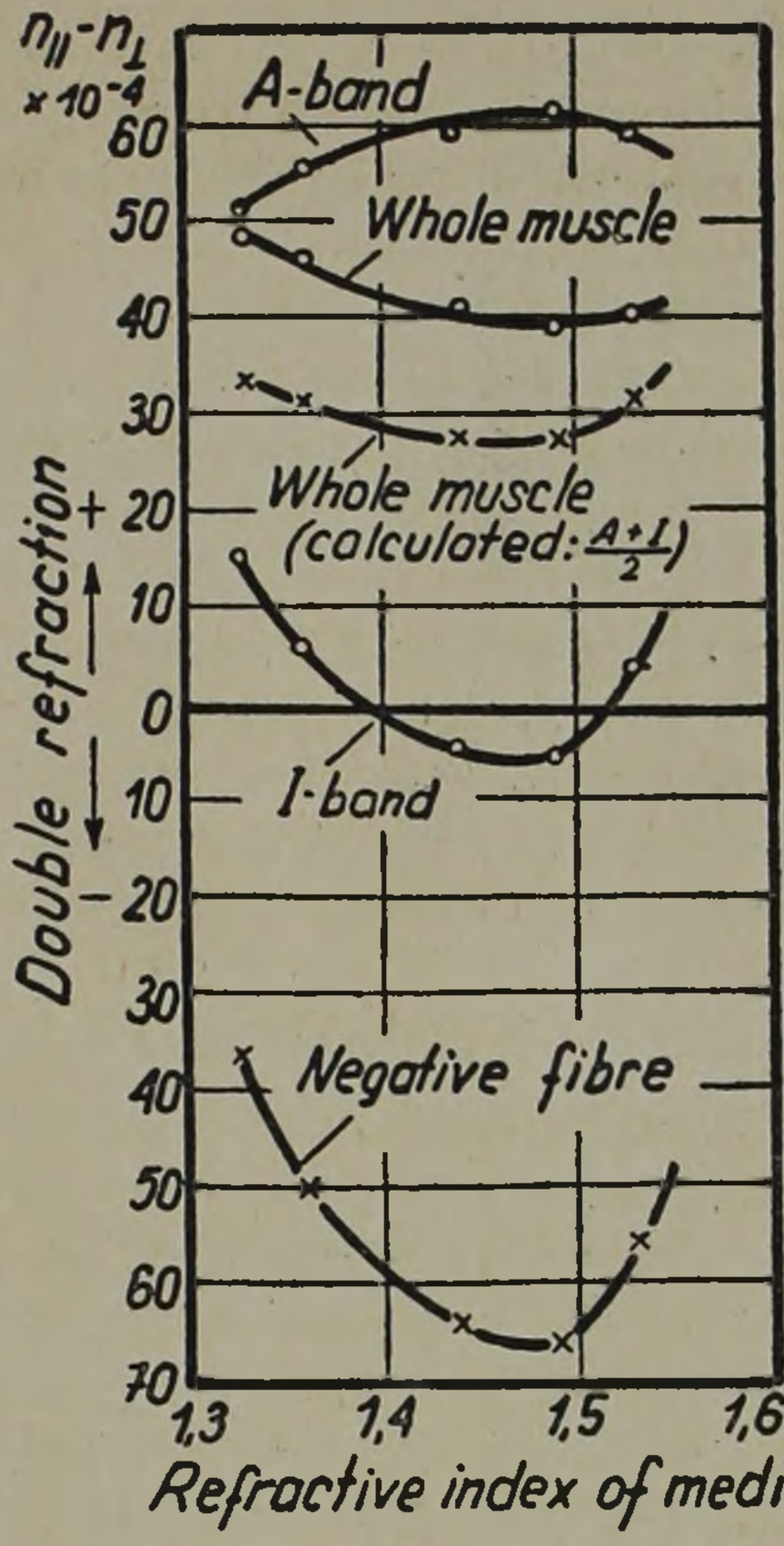

Figure 2.

The curve of the "whole muscle" as determined by us corresponds well with the curves known from the literature. The curve shows its own positive and the formal positive *DR* (Figure 2, Whole muscle).

The imbibition curve of the *A* band also curves upward here, hence it has its own positive and a formal negative *DR*. This corresponds completely with our results obtained by calculation. This curve is really the imbibition curve of the actomyosin measured in the *A* band (Figure 3, *A* band).

On the basis of geometrical analysis of these curves, the following can be stated:

The actomyosin filaments are present in the *J* band together with the *N*-protein. If, therefore, we subtract the curve of the actomyosin obtained in the *A* band from the curve of the *J* band, then we should get the *N*-protein curve. The curve obtained by calculation (*A*-band—*J*-band = Negative fibre) (Figure 2, Negative fibre) also corresponds well

qualitatively and quantitatively with the curve of the treated fibre containing *N*-protein,[9] which confirms our hypothesis that the imbibition curve of the *J* band is composed of the imbibition curves of the *N*-protein and actomyosin.

The *A* and *J* bands together contribute to the *DR* of the "whole muscle". So we must get the curve of the whole muscle from the mean-value of the curves of both bands. In reality the form of the curve calculated on the basis of the formula $\frac{A\ band\ +\ J\ band}{2}$ (Figure 2, Whole muscle, calculated) conforms well with the curve of the whole muscle found experimentally (Figure 2, Whole muscle) but it runs lower. The data of the curve run higher, however, if we take into consideration that the *A* bands are wider than the *J* bands, and moreover that the *Z* band also increases the positive *DR*. So the Whole muscle curve can be made in two ways: by adding the curves of the actomyosin and the *N*-protein (Figure 1), or on the basis of the mean-value of the *A* and *J* bands (Figure 2). The two processes mean fundamentally the same thing.

From the curve obtained from the *A* band conclusions of fundamental importance can be drawn. *According to the Wiener theory*[11] *the manifesting formal negative DR means that in the actomyosin a diagonal micellar structure dominates.*

As further support of our findings we investigated the imbibition curves of myosin, actin and actomyosin extraeted from the muscle and again stiffened into threads. Both the myosin and the actin — to the best of our knowledge — showed their own positive and a formal positive *DR;* the actomyosin, on the contrary, in these experiments also resulted in a mounting, arched curve, therefore its own positive and a formal negative *DR*. On the basis of our experimental results we can conclude that in uniting the actin and myosin into actomyosin a formation takes place in which some components are placed cross-wise.

SUMMARY.

We can state from calculations from the imbibition curve of the whole muscle and *N*-protein, that the imbibition curve of actomyosin must have its own positive and a formal negative *DR*.

This finding was also confirmed experimentally by determinations of the imbibition curve of the *A* band. From the form of the curve, according to the Wiener theory, we must conclude that one component of actomyosin is situated diagonally.

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THE P-CONTENT OF MYOSIN.[1]

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FROM THE INSTITUTE OF BIOCHEMISTRY, UNIVERSITY OF BUDAPEST.
(RECEIVED FOR PUBLICATION 20. 6. 47.)

According to K. Bailey (1) the P content of different preparations of myosin is between 0,048—0,067%. Treatment of the myosin at different pH-s, its dialysis and grade of hydration had no marked influence. Bate Smith (2) found similar values (0,04—0,06%). This suggests that the P is a building-stone of the myosin particle, which contains roughly one P for every 50,000 g.

According to the results of this laboratory "myosin" is rather a system than a homogenous substance. It consists of a fibrous sceleton and adsorbed globular particles. The enzymic activity is linked to these globules which are enzimatically active only in their adsorbed condition. They can be washed off from the sceleton by washing this latter at slightly acid reaction. On washing the composition of the "myosin" particle is changed and the question arises whether the P is actually a constituent of "myosin" and if so whether it is linked to the fibrous sceleton or the adsorbed globules.

In the present research crystalline myosin was used, prepared according to Szent-Györgyi, rendered free of actin by the method described by this author (3). As shown in this laboratory (4) the presence of actin makes the link between sceleton and globules stronger and prevents the separation of the two. This myosin was precipitated with alcohol, extracted three times with cold and three times with hot water, then with methyl alcohol containing 10% of benzol. Then the preparation was dried at 105° till constant weight and after being weighed it was boiled in a mixture of concentrated HNO_3 and H_2SO_4. After the HNO_3 was boiled off, the substance was further decomposed by the addition of H_2O_2. Finally the fluid was neutralised (indicator phenolphthaleine) and its P estimated according to Lohmann-Jendrassik.

[1] This research was sponsored by *Josiah Macy Jr. Foundation*, New York. Myosin prepared according to the method of Dubuisson-Horváth (4).

Part of the "myosin" preparation was treated, prior to its precipitation, by solvents, in ordrer to detach the globular proteins. This treatment was effected in two different ways, according to Banga and according to Guba (4). In the method of Guba the myosin is suspended in 0,02 m KCl of pH 6 under constant stirring for 24 hours at 0°. In Banga's method, which allows to regulate the degree of "inactivation" (detachment of the globules), 50% active myosin can be prepared in the following way: the myosin is dissolved in 0,5 m KCl containing 0,1 m pH 6 acetate-buffer. After storage for 24 hours at 0° the solution is diluted by water to a final KCl concentration of 0,04 m, and stored for another 24 hours at 0°. 1—2 mg of this myosin per ml. splits off in 10 min. 35—40% of the first labile P of 1,5 mg. ATP. If the myosin solution is allowed to stand at room temperature in 1 m KCl for 24 hours, diluted to 0,04 m KCl and stored for another 24 hours at the same temperature, the inactivation is complete.

The results were the following:

P content of active myosin in %	P content of inactivated myosin in %	Method of inactivation
0,057	0,039	Guba 24h
0,057	0,025	Guba 48h
0,079	0,058	Guba 24h
0,079	0,046	Banga 90%
0,077	0,047	Guba 24h
0,062	0,036	Guba 24h
0,062	0,025	Banga 90%
[1]0,035	0,019	Guba 24h
[1]0,035	0,024	Banga 50%
[1]0,035	0,025	Guba 14h
[1]0,035	0,017	Banga 100%

These results show that fully active "myosin" contains on average 0,056% P, which is in good agreement with previous authors. On treatment of this myosin with different solvents this P content drops to 40—60% of its original value, which shows that the P, found in full myosin, is not a constituent of the sceletal substance, or is so only partially, the former assumption being the more probable. It is either a constituent of the globular proteins or is a constituent of other adsorbed substances.

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STRIATION IN ACTIN SOLUTIONS.

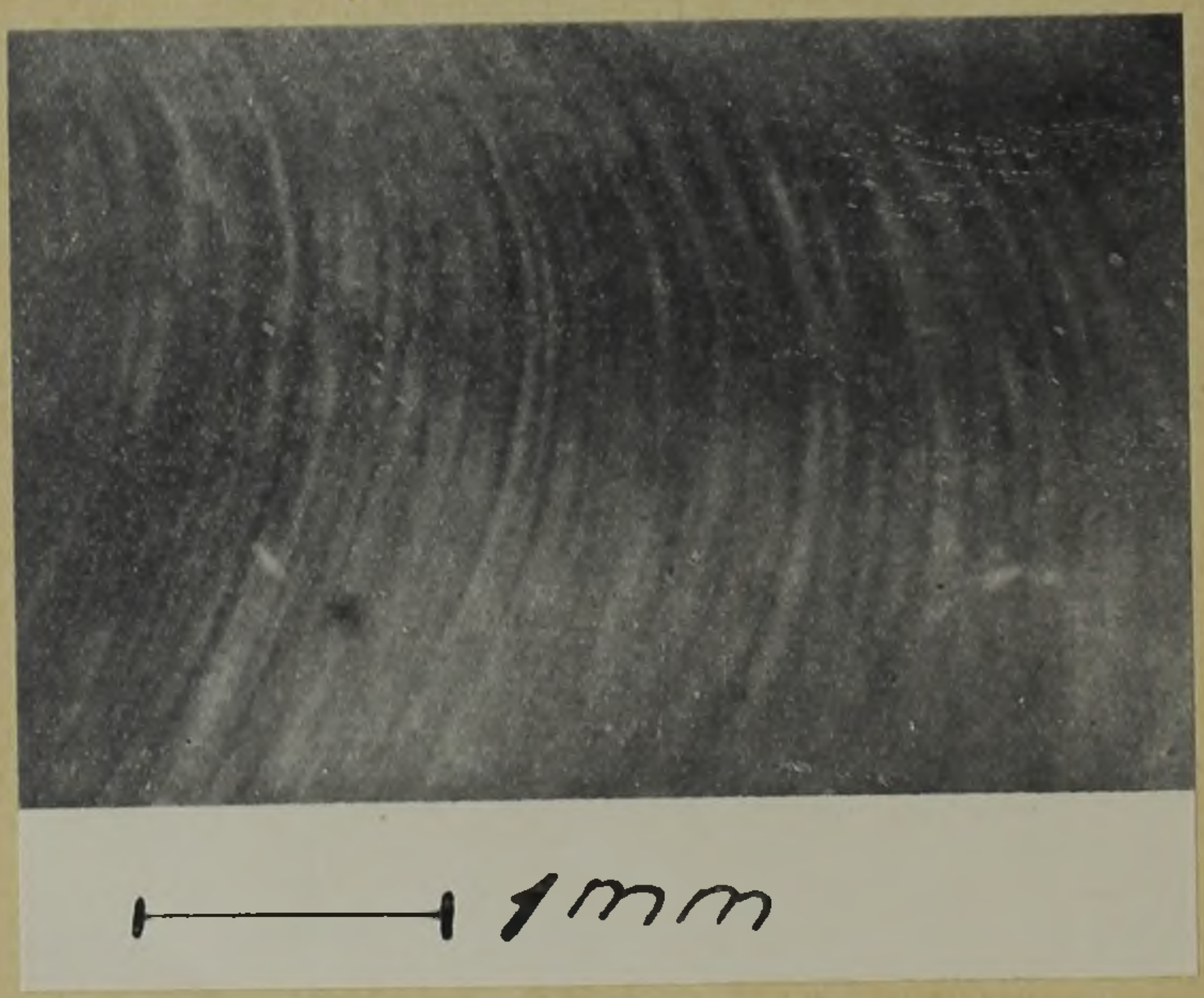
WITH 1. FIG. IN TEXT.

BY L. VARGA.

FROM THE INSTITUTE OF BIOCHEMISTRY, UNIVERSITY OF BUDAPEST.

(RECEIVED FOR PUBLICATION 23. 6. 47.)

Matoltsy and Gerendás have shown that the isotropy of I discs in cross striated muscle is due to the presence of a negatively double-breaking protein which compensates the positive double-break of the actomyosin fibre. The question arises why this protein has a periodic distribution.



Actin solution, agitated by a glass-rod. Polarisation microscope. Magn. 10.

G. v. Iterson has shown (1) that fibrous colloids assume periodically changing properties under influence of mechanic stress. The same has been shown in the case of Tobacco Mosaic Virus by Bernal and Fankuchen (2).

The author has found that strong solutions of fibrous actin are especially suited for the study of this phenomenon. If actin is precipi-

tated in its isoelectric point (3), and is dissolved in a small vol. of 0,1 m Na_2CO_3,[1] the resulting fluid shows under the polarisation microscope a splendid striation if exposed to gentle agitation. Pulling a glass-rod through the solution suffices to produce such a structure which is stable for 30 minutes-24 hours (see figure). The author hopes to promote the study of this phenomenon by drawing attention to actin as an especially favourable material.

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[1] Excess has to be avoided since alkaline reaction destroys actin.

ACTIVATION ENERGY OF THE CONTRACTION OF ACTOMYOSIN.*

WITH 2 FIG. IN TEXT.

BY L. VARGA.

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(RECEIVED FOR PUBLICATION 17. 6. 47.)

It has been shown in a previous paper[1] that the contracted and relaxed state of actomyosin are two distinct physical states with no transitory forms. A single particle is either fully contacted or fully relaxed and a partial contraction of actomyosin is not due to the partial contraction of single particles, but to the complete contraction of part of the particles. It has also been shown that this reaction, the transition of relaxed (*R*) into contracted (*C*) actomyosin is an equilibrium reaction and that the equilibrium between *R* and *C* particles is a function of temperature. At o° there is no contraction at all while at 16° contraction

temperature	minutes	Relaxed actomyosin particles % in washed muscle slices									Relaxed actomyosin particles % in actomyosin threads								
		0	0,5	1	1,5	2	2,5	3	4	5	0	0,5	1	1,5	2	2,5	3	4	5
		100	—	78	—	66	50	39	—	—	100	—	81	—	54	—	46	38	—
		100	—	82	—	64	—	45	—	—	100	86	79	—	—	50	46	—	—
	5°	100	—	—	65	58	50	45	—	—	100	89	80	—	59	54	51	38	—
		100	—	—	65	52	—	47	—	—	100	89	—	65	60	—	—	39	—
		100	—	—	65	58	—	48	—	—	100	—	80	—	—	49	46	—	—
		100	15	56	50	—	—	—	18	—	100	68	—	50	—	32	—	—	—
		100	—	—	48	—	34	25	—	—	100	—	60	—	36	—	25	—	—
	10°	100	—	60	45	—	32	—	17	—	100	—	—	50	—	32	—	18	—
		100	15	—	45	40	—	25	—	—	100	72	—	50	—	32	—	—	—
		100	—	58	—	38	32	—	18	—	100	—	63	44	38	—	—	17	—
		100	62	40	—	24	—	—	—	—	100	63	42	30	22	—	—	—	—
		100	62	46	—	20	—	—	—	—	100	—	43	32	—	—	—	—	—
	15°	100	62	—	—	18	—	—	—	—	100	65	45	32	—	—	—	—	—
		100	—	35	—	20	—	—	—	—	100	—	—	32	18	—	—	—	—
		100	—	40	—	18	—	—	—	—	—	—	—	—	—	—	—	—	—

* This research was sponsored by the *Josiah Macy Jun. Foundation*, New York.

is nearly maximal, that will say practically all particles are in the contracted state. Between these two limits to every temperature an equilibrium of R/C can be correlated. This equilibrium can be calculated from the final length which the system reaches at the specified temperature on contraction — be it in a muscle fibre or in an actomyosin thread.

In the previous research the final equilibrum length has been measured at different temperatures, no attention being payed to the rate of contraction, *i. e.* the $R—C$ transition. It has been shown that contraction is an endothermic process taking up 56,000 cal. from its surrounding, while the free energy of the system drops by 7000 cal. (37°).

In the present research the rate of contraction, *i. e.* the rate of the reaction $R—C$ has been measured at different temperatures. The rate of such a reaction allows to draw conclusions on the nature of the process and its energy of activation. The temperatures were: 5°, 10°, and 15°.

The method employed was the same as in the previous experiments.[1] The suspension fluid had the following composition:

aqu. dest.	0,8 ml.,
0,5 m *KCl*	0,1 „
1,2% *ATP*	0,1 „

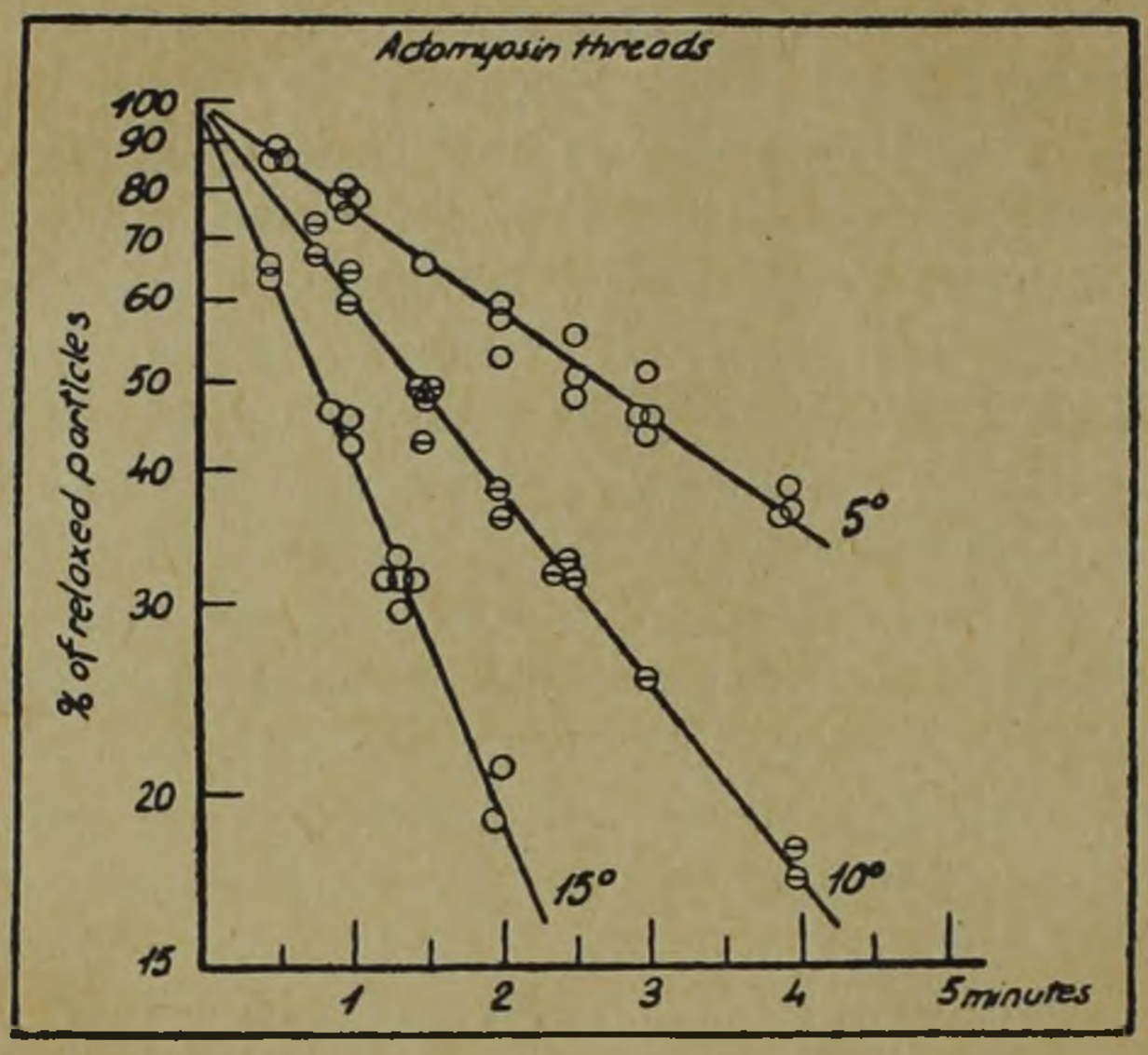
The results are summed up in the two figures and the table. (Ordinate in logaritmic scale.) The curves show that the $R—C$ transformation is a monomolecular reaction since the log. of the shortening, as function of time, is a straight line. The half-time increases by the factor of 4 for every 10 degrees. From the data of the Tab. the energy of activation can be calculated. Since the reaction is monomolecular the half-time can be used instead of the constant of the rate of the reaction. The activation energy E is thus:

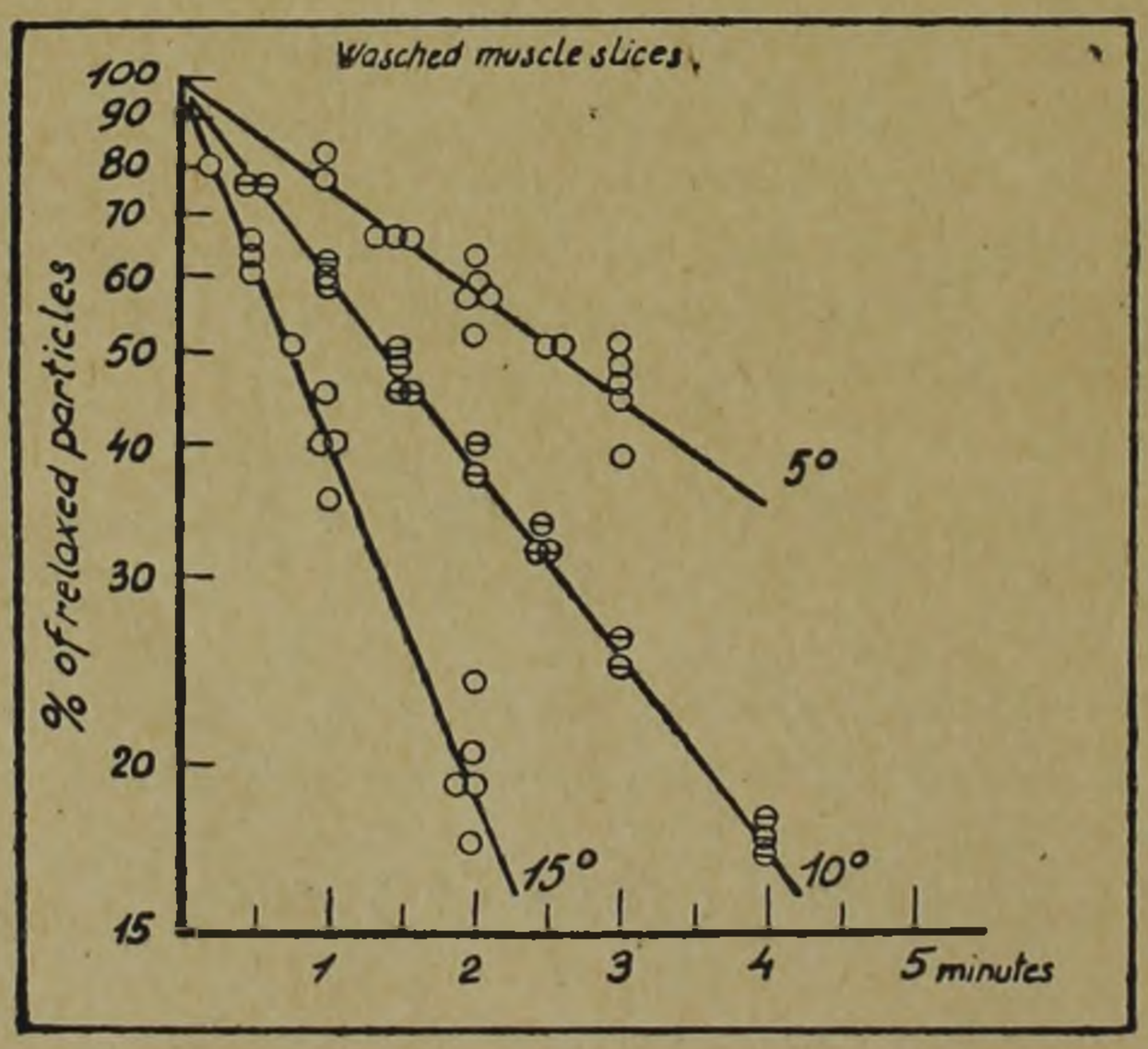
$$E = \frac{2{,}303\ (log\ t_{(f)1} - log\ t_{(f)2})\ R.T_1.T_2}{T_1 - T_2}$$

where $t\ (f)$ is the half-time, R the gas constant, T the abs. temp. According to the table and the figures between 5—15°

$$E = \frac{2{,}303\ (log\ 2{,}60 - log\ 0{,}75)\ 1{,}986\ .\ 80{,}000}{10} \cong 20{,}000\ cal.$$

This value of 20,000 cal. is in good agreement with the data of Speakman[2] found for E of the $\alpha—\beta$ transformation of keratin. The acti-


Actomyosin threads
% of relaxed particles
100
90
80
70
60
50
40
30
20
15
1
2
3
4
5 minutes
5°
10°
15°


Washed muscle slices
% of relaxed particles
100
90
80
70
60
50
40
30
20
15
1
2
3
4
5 minutes
5°
10°
15°

vation energy of threads or muscle fibres (slices) is identical. It is not probable thus that the activation energy should be considerably dependent on structure, nor that diffusion plays an important role in our experiment. This later possibility is also eliminated by the found relation of rate and temperature. The numeric value of E makes it improbable that the activation energy should be needed to break one strong link. It is rather probable that this energy is needed to break a number of weaker links, like H-bonds.

My thanks are due to professor Laki for his interest, help and encouragement.

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ANTIBIOTIC EFFECT OF *d*-AMINOACIDS.

WITH 2. FIG. IN TEXT.

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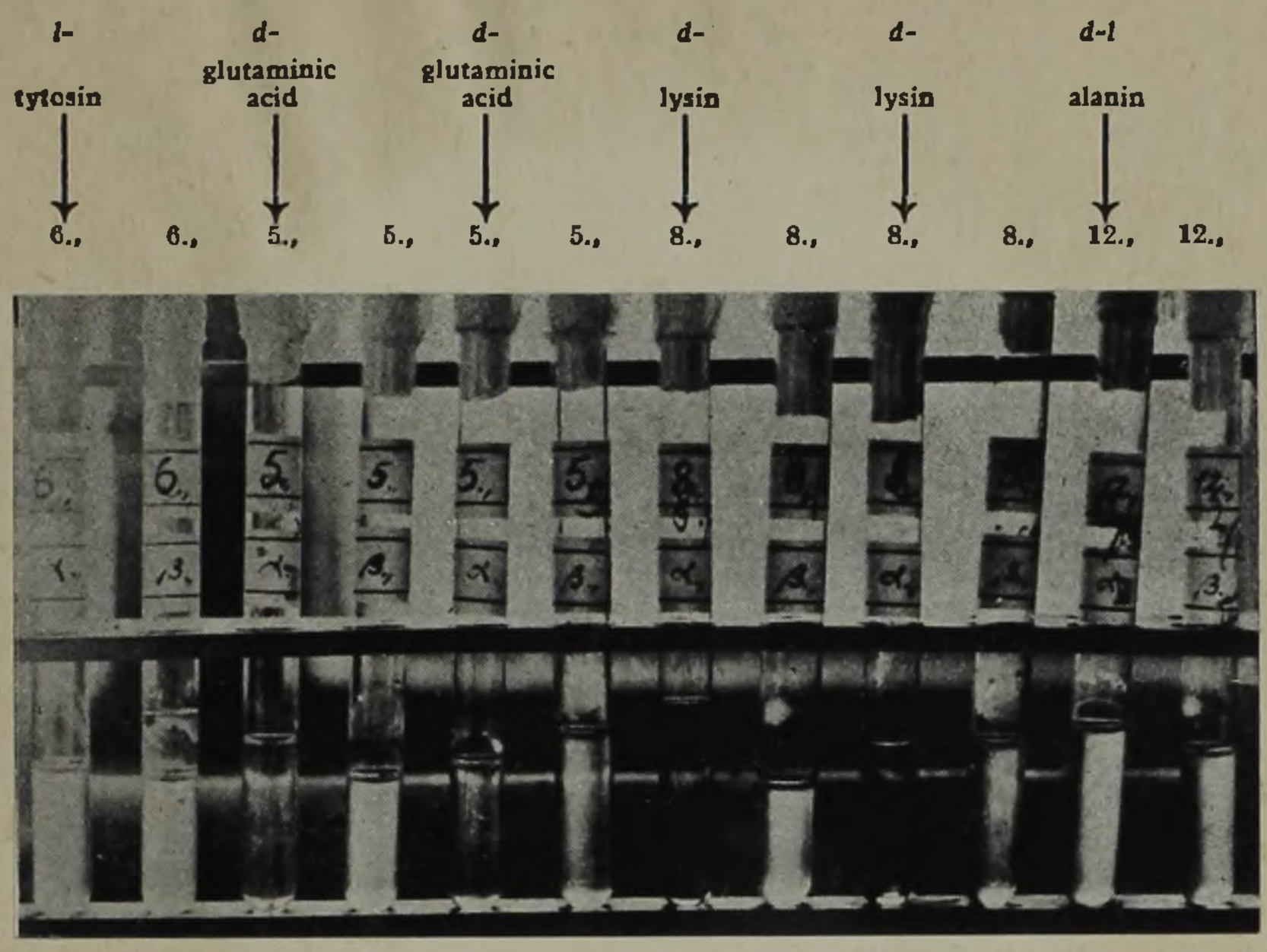
FROM THE INSTITUTE OF HYGIENE OF THE UNIVERSITY OF DEBRECEN.
(RECEIVED FOR PUBLICATION 27. 6. 1947.)

Ivánovics and *Bruckner* (1937) (6), (7), (8), in investigating the chemical nature and immunbiological behavior of the capsulesubstance of anthrax-bacilli had demonstrated, that the higher organisms are not capable to disintegrate the "not natural" polypeptids of this capsule-substance, which is purely composed from d-glutaminic acid. The organism cannot produce "protectiv ferment" (Abderhalden) against this polypeptid. *Kögl* and *Erxleben* (1939) (9), in the proteins of carcinomatous tumors found also chiefly d-aminoacids.

We know now, that the penicillin is composed similarly from two "not-natur*al*" d-aminoacids, the $\beta\beta$-dimethylcystein and serin. It was for this reason, that I got interested in the question as to whether also other aminoacids from the d-series (fortuitously at my disposition) can exert the same effect on penicillin-sensible microorganism or not. As nutrient-medium was used a salt-mixture with glucose. (Sodium chloride 0,9 g, Potassium chloride 0,042 g, Calcium chloride 0,024 g, Sodium bicarbonate 0,02 g, Magn. sulfat 0,04 g, Disodium phosphate 0,04 g, Ferrosulfat 0,04 g, Glucose 3,00 g, Water 100 g.) In one part of the test-tubes ("α"-tubes) 1 millil. of n/100 aminoacid was added to 10 millil. of nutrient-medium, the β-tubes contained no aminoacids, they served as controls. Each tube was inoculated with 2 drops of an emulsion of Staphylococcus aureus (Oxford-H) and incubated at 37 C°. After 24 hours in the test-tubes "5, α" and "8, α", which contained d-glutaminic acid and d-lysin, the staphylococci after incipient multiplication became dissolved and settled down. The supernatant fluid became clarified. In the test-tubes with l-tyrosin and with racemic d-l-alanin (6, α, and 12, α tubes) this phenomenon was not to be observed. In these tubes ("α") the multiplication of the cocci was the same as in the control-tubes ("β"). (Figure 1.)

In further experiments agar-plates were evenly inoculated with a fresh emulsion of staphylococci (Oxford-H strain). In the Heatly-tubes



I. Nutrient medium : salt mixture with glucose.
II. Inoculaton: with 2 drops of an emulsion of Staphylococcus aureus (Oxford-H)
III. Incubation: 24 hours at 37° C
α = tubes with aminoacids $\left(1 \text{ millil.} \frac{n}{100}\right)$; β = no aminoacids

penicillin (10—50—100 O. U.) and aminoacids from the d-series (0,1 milliliter of $\frac{n}{100}$ d-glutaminic acid, d-lysin, d-arginin) was distributed. After 24 hours incubation the inhibition of bacterium-growth around the tubes with penicillin was characteristic and expressed. Around the tubes with aminoacids was also a small zone of inhibition, more expressed around the tubes filled with d-glutaminic acid and d-lysin (tubes 4, and 5). (Figure 2.)

Discussion : According to these results the antibiotic and bacteriolytic effect of the d-aminoacids can be regarded as an established fact. One may assume, that d-dimethylcystein and d-serin, components of the penicillin, liberated in the organism are taken up in the proteinstructure of the bacteria, "unnatural" proteins are produced. Perhaps that is the reason why the bacteria after treament with penicillin are not capable to multiply and giant-cells are formed. One may assume too, that from the disintegrated bacteria these d-aminoacids became liberated again and can enter into another bacterium individuum, in which the

Inoculation of agar-plate with Staphylococcus aureus. (Oxford-H strain.)

same process will be repeated. With a chainreaction like that one might explain the observation, that-contrary to the mode of action of the sulfonamids- with comparatively small dosis of penicillin a great many bacterium cell can be inhibited and dissolved. A similar process might perhaps account for the action of bacteriophages. It is on that way, that the "lytic agent" with filtrates free from bacteria can ba transmitted in series. The α-aminoacids of gramicidin in 45% belong to the d-series *(Hotchkiss R. D.* and *Dubos,* 1940—41) (2, 3, 4, 5), 20% of the α-aminoacids of tyrocidin are represented also by d-aminoacids.

(*Lipmann, Hotchkiss* and *Dubos,* 1941) (10) *Fox S. W., Fling M.* and *Bollenback G. M.* (1944) (1), succeeded in inhibiting the growth of bacteria with d-leucin. Further experimentes along this line are inviting.

Summary: Glutaminic acid and lysin, both from the d-series can exert an antibiotic effect on staphylococci. One may assume, that the two d-aminoacids of penicillin liberated in the organism are responsible for the action of the later. A similar process can be suspected in the action of the bacteriophages.

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CORRECTION OF THE FAILURE OF HEAT TOLERANCE OF THYROIDECTOMIZED ANIMALS WITH THERMOTHYRINE.

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(RECEIVED FOR PUBLICATION ON 15. 9. 1947.)

In earlier studies[1],[2] concerning body temperature of thyroidectomized animals in a hot environment it was shown, that heat tolerance is reduced by thyroidectomy.

These experiments were carried out on pairs of guinea pigs with equal heat tolerance. Animals of the same growth, weight and sex were overheated in a thermostat of 34—35 C° during several hours. In a great number of preliminary experiments we were able to find pairs with practically identical body temperature curves. As a numerical expression of the hyperthermia we used the „time-temperature-area" in our statistical and error calculations. The time-temperature-area is the area inclosed by the hyperthermic body temperature curve and the horizontal axis in a coordinate system, measured planimetrically and expressed in mm^2. Since the size of the time temperature area depends on height and duration of the hyperthermia i. e. both factors characterising heat tolerance, it seems to form the most reliable basis for evaluation. If in a number of preliminary experiments a pair of guinea pigs have shown nearly the same time-temperature-areas i. e. nearly the same heat tolerance, one of them was thyroidectomized. The thyroidectomized member of the pair reacted to overheating with greater time-temperature-area than the control: its body temperature rose in the thermostat higher and for a longer period than that of the normal, signifieing a reduced heat tolerance of the thyroidectomized animal. As administration of thyroxine did not improve the heat tolerance of thyroidectomized guinea pigs — on the contrary: their time-temperature-areas increased during thyroxine administration still further — lack of thermothyrine *A* was suggested as an explanation.

As *Mansfeld*[3] demonstrated the heat protective hormone — thermothyrine A or cooling hormone — is always poured out by the thyroid when danger of hyperthermia arises, and reduces heat production and

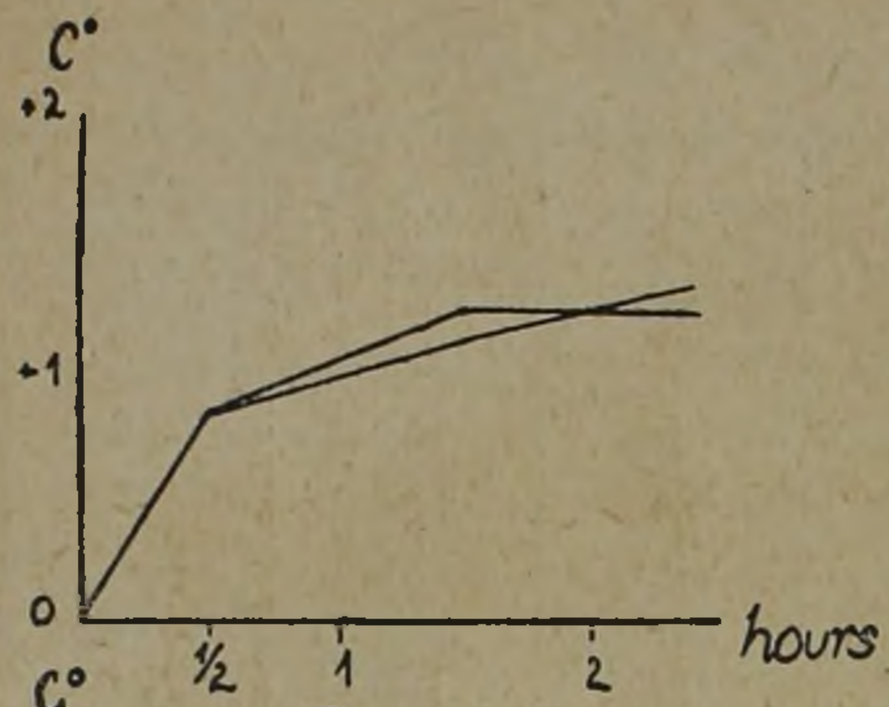

Fig. 1.

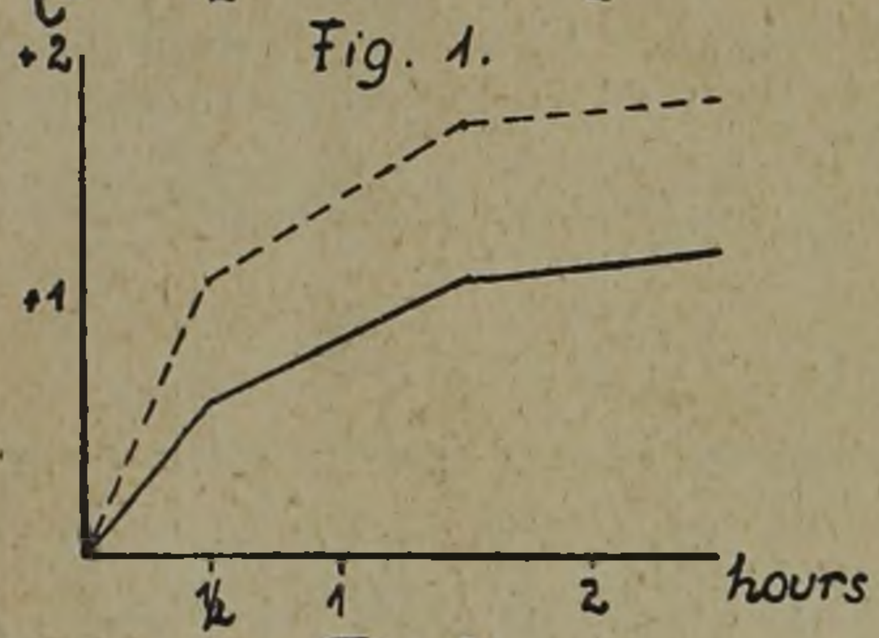

Fig. 2.

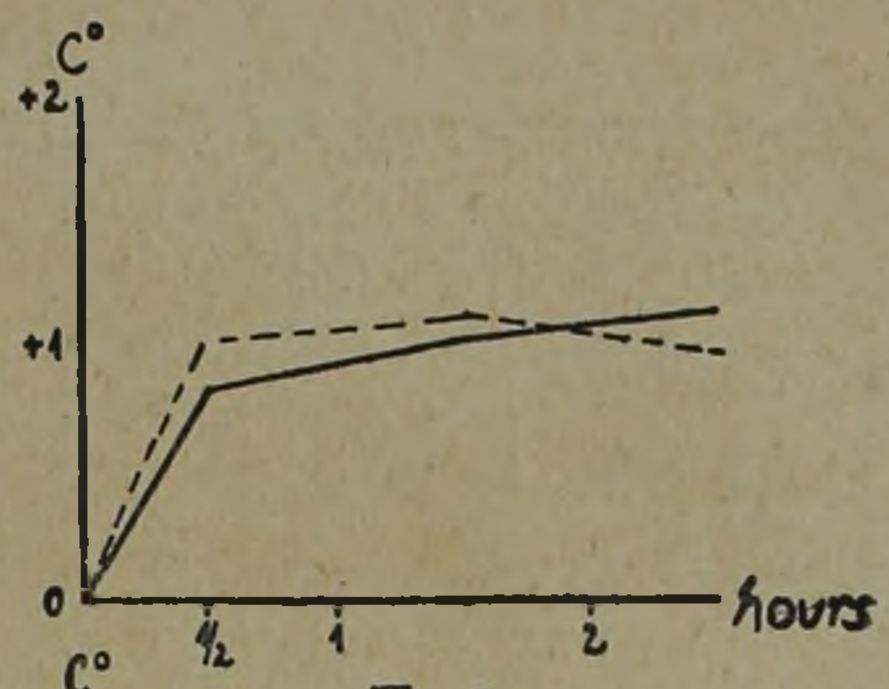

Fig. 3

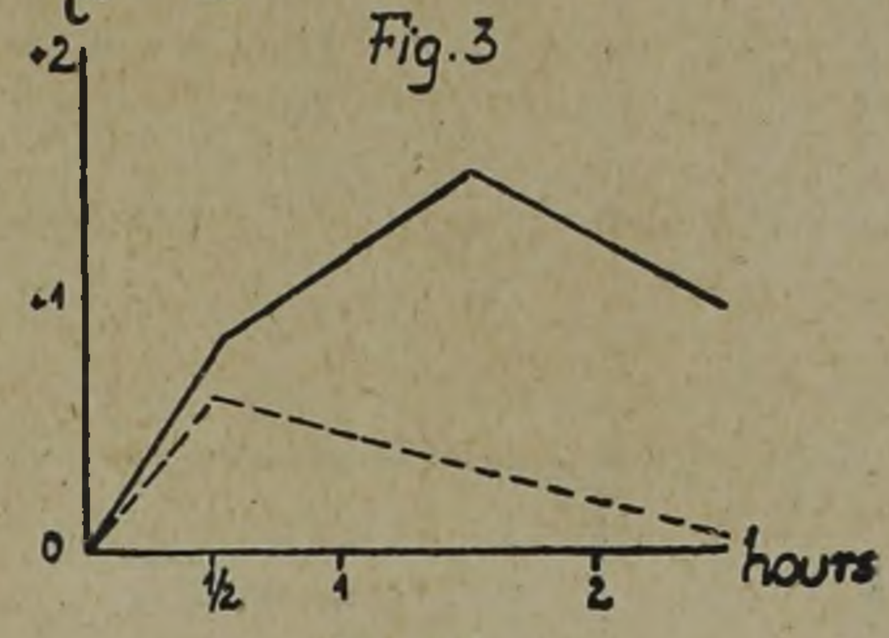

Fig. 4.

alters the sensitivity of the organism to thyroxine. (Thermothyrine is not identical with the thyreogen factor influencing haemopoesis[4] and its secretion can be inhibited like the secretion of thyroxine by methylthiouracil.[5 6])

In experiments reported in this paper we tried to correct the disturbed heat tolerance of thyroidectomized guinea pigs by administration of thermothyrine. We used a fraction of hydrolysed summer-thyroid called "thermothyrine total", free of thyroxine, but containing both thermothyrines *A* and *B*.[3] 1 cc of this solution corresponded to 5 grm thyroid gland. It is a pleasure for me to acknowledge, that this praeparation was produced and placed at my disposal by Mrs. *A. Mansfeld—Oppenheim* and to express my best thanks for here courtesy.

Fig. 1. shows an example of body temperature curves and time-temperature-areas of two guinea pigs, which — in several overheating experiments — have shown equal heat tolerance. Fig. 2. shows an example of an overheating experiment of this pair after thyroidectomy was performed on one animal. The body temperature curve of the thyroidectomized animal (dotted line) rose higher, its time-temperature-area is greater i. e. its heat tolerance is less than that of the control. Fig. 3. and 4. demonstrate experiments in which the thyroidectomized member of the pair was treated with thermothyrine. 1 to 3 cc "thermothyrine total" solution

TABLE 1.

Guinea pigs	Nr. 1. (normal)	Nr. 2. (normal)	Nr. 1. (normal)	Nr. 2. (thyroidectomized)	Nr. 1. (normal)	Nr. 2. (thyroidectomized + thermothyrine)
Time-temperature-areas:	1040	1120	1200	1580	1110	1200
	1210	1190	970	1400	960	1100
	1300	1100	980	1050	950	870
	970	1250	1070	1290	930	1000
	1130	1220	1090	1300	1210	1190
	1280	1100	1000	1370	1010	890
	$\overline{x'}$ 1163		$\overline{x'}$ 1331		$\overline{x'}$ 1041	
	$\overline{x''}$ 1155		$\overline{x''}$ 1051		$\overline{x''}$ 1028	
	$P > 0{,}05$		$0{,}01 > P > 0{,}001$		$P > 0{,}05$	

TABLE 2.

Guinea pigs	Nr. 3. (normal)	Nr. 4. (normal)	Nr. 3. (normal)	Nr. 4. (thyroidectomized)	Nr. 3. (normal)	Nr. 4. (thyroidectomized + thermothyrine)
Time-temperature-areas:	1270	1090	1100	1370	1170	1290
	1120	1240	1210	1480	930	1120
	960	1080	990	1450	1310	1220
	870	1110	890	1270	950	1110
	1190	990	1170	1390	1170	1200
	1370	1280	1210	1450	1350	1090
	$\overline{x'}$ 1131		$\overline{x'}$ 1401		$\overline{x'}$ 1171	
	$\overline{x''}$ 1130		$\overline{x''}$ 1095		$\overline{x''}$ 1130	
	$P > 0{,}05$		$P < 0{,}001$		$P > 0{,}05$	

TABLE 3.

Guinea pigs	Nr. 5. (normal)	Nr. 6. (normal)	Nr. 5. (normal)	Nr. 6. (thyroidectomized)	Nr. 5. (normal)	Nr. 6. (thyroidectomized + thermothyrine)
Time-temperature-areas:	1370	1240	1370	1640	1300	1400
	1520	1240	1290	1490	1280	1100
	1350	1470	1250	1680	1530	380
	1600	1510	1310	1710	1370	970
	1420	1290	1320	1510	990	1000
	1310	1420	1320	1400	1430	1200
	$\overline{x'}$ 1428		$\overline{x'}$ 1571		$\overline{x'}$ 1316	
	$\overline{x''}$ 1356		$\overline{x''}$ 1290		$\overline{x''}$ 1008	
	$P > 0{,}05$		$P < 0{,}001$		$P > 0{,}05$	

were injected subcutaneously 3 to 4 hours before overheating was begun. It is evident, that the time-temperature-area of the thyroidectomized guinea pig is not greater than that of the control; the disturbed heat tolerance is restituted. Sometimes even overcompensation was observed and the thermothyrine treated thyroidectomized animal was even less sensitive against overheating than the control. (Fig. 4.)

Full details of these experiments can be found in tables 1 to 3. It is evident and prooved with statistical and error calculations,[7] that there was no significant difference between the time-temperature-areas of the guinea pig pairs before thyroidectomy i. e. animals with equal heat tolerance were choosen. After thyroidectomy the time-temperature-areas of the operated animals becam significantly greater i. e. their heat tolerance decreased. If these animals were treated with "thermothyrine total" the difference between the time-temperature-areas disappeared, thermothyrine reestablished normal heat tolerance. In some cases even the values of the thermothyrine treated thyroidectomized animals are significantly below the controls i. e. the heat tolerance of the thermothyrine treated guinea pigs exceeds that of the normals.

SUMMARY.

Thermothyrine raises the reduced heat tolerance of thyroidectomized guinea pigs to a normal level, while thyroxine reduces heat tolerance even further.

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STUDIES ON THE COMPOSITION AND POLYMERISATION OF ACTIN.

(WITH 11 FIGURES IN TEXT)

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(RECEIVED FOR PUBLICATION 11. 10. 47.)

INTRODUCTION.

In previous communications the preparation and some properties of actin were described (1, 2). Experimental evidence was presented to show that this protein, prepared from the water insoluble part of the muscle fiber, plays an important role in the chemical mechanism of muscle contraction (3).

The most striking properties of actin are: 1. that it may exist in a globular form, which on addition of inorganic ions changes into a highly viscous, fibrous one, 2. that its fibrous form, when added to myosin, gives the actomyosin, which — according to experimental conditions — is precipitated or dissociated by adenosine triphosphate. Threads of actomyosin imitate muscle fibers, as they contract in presence of adenosine triphosphate and inorganic ions.

In order to understand the physiological role of actin, two problems must be solved. First, we have to know the nature of the forces linking actin and myosin together, and the influence of adenosine triphosphate upon these. Second, we have to know the detailed mechanism of the reaction, which changes globular actin into fibrous actin, in other words, the polymerisation of actin. That this change is a real polymerisation is confirmed by the study of *Jakus* and *Hall* (4).

Both of these problems require the close chemical investigation of actin.

Actin has not yet been obtained in a pure form, although we have every reason to suppose that our preparations do not contain appreciable amounts of impurities. None of the procedures used in protein chemistry for the purification of proteins lead to any improvement in the homogeneity of actin, and most of them cannot be applied without denaturation of the protein. As described in the present paper, part of our failure

must be ascribed to the presence of a prosthetic group and to the easy oxidizability of actin.

It appears from our present studies that the easily oxidised groups of actin, together with *Mg* are responsible for the polymerisation. It is possible that the same groups are involved in the reaction between actin and myosin.

PREPARATION OF ACTIN.

Throughout this work we have used the methods outlined by *Straub* (2) and modified by *Guba* and *Szent-Györgyi* (3). During the summer season we have encountered difficulties in the preparation. After a study of the effect of temperature during the various phases of the procedure, we have further specified the conditions for the method of preparation. Although most of the operations were previously described, we want to summarise the procedure as presently adopted.

1. The rabbit is killed by decapitation, rapidly skinned, eviscerated and packed in ice. After a few minutes, the muscles from the legs, the back and the abdomen are cut off and minced through an ordinary meat chopper.

2. Every 100 g of the mince are suspended in 300 ml ice cold 0,3 *M KCl* containing 0,15 *M K*-phosphate buffer of *pH* 6,5. The temperature is kept at 0—5 C⁰ and the mixture is constantly stirred. After 10 minutes, 1200 ml glass distilled water are added for every 100 g muscle and the mixture is immediately pressed through a thin cloth.[1]

3. The remaining muscle tissue is suspended in 5 volumes of a 0,4% $NaHCO_3$ solution of 22—25 C⁰ and kept at this temperature with constant stirring for 30 minutes. This fluid is again removed through a thin cloth.

4. The residue is suspended in one volume of a cold solution containing 0,01 *M* $NaHCO_3$ and 0,01 *M* Na_2CO_3. The temperature should be kept below 10 C⁰, the mixture is thoroughly stirred. After 10 minutes the mixture is diluted with 10 volumes of glass distilled water of 22—25 C⁰ and the fluid is squeezed through a thin cloth as fast as possible.

5. The residue is weighed and for every 100 g of it 300 ml of acetone are added. Temperature 22—25 C⁰. Having stirred it for 10 minutes, the acetone is gently pressed out through a thin cloth and $^1/_3$ of the former amount of acetone is added again, stirred for 10 minutes, pressed down and the residue is dried in air at room temperature.

[1] The fluid may be saved for the preparation of myosin, according to *Szent-Györgyi* (5).

6. After 5—10 hours of drying, actin may be extracted from the dry muscle. 1 g of dry powder is treated with 20 volumes of CO_2-free glass distilled water for 15—20 minutes at room temperature. The solution of actin is sucked off through a Buchner funnel. It contains 4—8 mg actin per ml.

The dried muscle preparation is stable for at least a week, it keeps well at low temperature in a desiccator.

Notes. It is most important to observe the prescribed temperature during each of the operations. Washing the muscle residue with alkaline solutions leads to its moderate swelling. In these cases the fluid is pressed out only so far, until it runs out smoothly under moderate pressure and it does not become turbid or viscous. The acetone should be cooled below 20 C°, otherwise the mixture becomes too warm. We have found that the yield of the actin is increased if 0,0001 *M* $CaCl_2$ is added to the washing fluids in steps 3 and 4. Addition of the same amount of $MgCl_2$ decreases the yield.

The actin solutions thus obtained are usually more or less cloudy depending on the state of nutrition of the animal. The cloudiness results from the presence of lipids, which were not removed by the acetone treatment. Repeated extraction of the dry muscle with acetone will remove them. This is best accomplished in a Soxhlett apparatus, if solid granulated $CaCl_2$ is added to the extracting acetone to fix the residual humidity, the dried muscle can be refluxed with boiling acetone more than 10 times, without destroying its actin content. The actin solutions obtained from exhaustively extracted dried muscle are completely limpid and colourless.

If a salt free actin solution is diluted with ten volumes of acetone and then a few drops of an acetate buffer (*pH* 4,6) are added, the actin is precipitated, while the lipid impurities remain in solution. Dissolving the precipitate in water, a concentrated solution of globular actin is obtained.

CHARACTERISATION OF ACTIN.

Actin was defined as a protein, which combines with myosin to form actomyosin, a compound characterised in its turn by its peculiar behaviour towards adenosine triphosphate. A quantitative estimation of actin based on this property was outlined by *Straub* (2). This is however too complicated for routine measurements. We have therefore detected actin mostly by measuring the viscosity of its solution, after the addition of salt has effected its polymerisation. The "viscosity" of actin is naturally only an apparent viscosity, as its solutions are non-Newtonian. The apparent viscosity of an actin solution decreases with higher pressure, but does not reach a constant value even at 100 *Hg* mm pressure. However,

at higher pressure the error in the viscosity measurements, introduced by small changes in the pressure and by the thixotropy of the solution, is greatly reduced. We decided to measure the viscosity of actin at the arbitrary pressure of 60 *Hg* mm.

The solution was run through a horizontal capillary of 200 mm length, diameter 0,3 mm, on each end of which horizontal tubes of 2,5 mm diameter served to hold the amount of fluid to be investigated. (Usually 4,5 ml.) The length of these tubes was 100 cm on each sides, at one end it was connected with a three way cock, through which the system could be connected with a 12 l flask of air, compressed to the desired pressure. The time required to move the meniscus 80 cm along the horizontal tube, was about 50 seconds in case of water at 24 C^0, when the pressure was 60 *Hg* mm. The horizontal tubes were centered in a water bath of 50 mm diameter.

At 60 *Hg* mm pressure and 24 C^0, the apparent specific viscosity of actin solutions after polymerisation was found to be

$$\frac{\eta_{sp}}{mg\ N/\ ml} = 1{,}7$$

for specific viscosity values up to 1,0.

AMINO ACID COMPOSITION OF ACTIN.

Some of the amino acids were quantitatively estimated in our actin preparations by *L. Borsodi.* His results are briefly summarised in Table I. A different sample of actin preparation was used for each determination.

The low tryptophane and arginine content, the high aspartic acid and lysine content, with relatively high cystine content are characteristic of actin. The absorption spectrum of actin is reproduced in Fig. 1. It is in good agreement with the analytical data. Polymerisation of actin on addition of salts does not lead to any appreciable change in the absorption spectrum.

From the tryptophane content of actin, its minimum molecular weight is more than 70,000. As a solution of globular actin just passes a 10% collodion membrane (prepared according to Bechhold) its molecular weight cannot be higher.

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TABLE I.

Amino acid content of actin.

Amino acid	Amino acid N. as % of total N	Average	Method	Amino acid	Amino acid N. as % of total N	Average	Method
Tryptophane	0,23		1	Glutamic acid ...	5,36		8
	0,21				5,50		
	0,23				5,48		
	0,20	0,22				5,49	
					5,62		9
Tyrosine	1,37		2				
	1,45			Aspartic acid	11,52		8
	1,51				10,78		
	1,47	1,45			10,60	10,97	
Phenylalanine	none		3	Proline	5,00		10
					4,94		
Arginine	1,58		4		5,18		
	1,63				5,19	5,08	
	1,62						
		1,60		Hydroxyproline ..	1,19		10
	1,65		5		1,26	1,22	
Histidine	2,46		6	Glycin	8,38		11
	2,47	2,46			8,15		
					8,32		
Lysine	11,85		5		8,35	8,30	
	11,55						
	10,95	11,48		Methionine	none		*
Cystine	1,37		7	Amide — N	11,5	11,50	
	1,45						
	1,51			Undetermined — N		40,0	
	1,41	1,44				Total: 100,0	

CALCIUM CONTENT OF ACTIN.

Actin preparations invariably contain a constant amount of calcium. We have found that the *Ca* content of actin does not change even on prolonged dialysis. The close connection between actin and *Ca* is evident if we compare the *Ca* and *Mg* content of the dried muscle preparation with those of the actin solutions prepared from it. Repeated washing of the isoelectric precipitate of actin with dilute acetate buffer of *pH* 5, does not reduce significantly its *Ca* content.

The *Mg* content of the actin preparation is variable and small. Yet *Mg* seems to be indispensable for the formation of polymerised actin, whereas the role and significance of the presence of *Ca* in globular actin remains unknown.

* The absence of methionine was inferred from the fact that the sum of cystine S and inorganic S (0,08%) is equal to the total S content (0,62%) of actin.

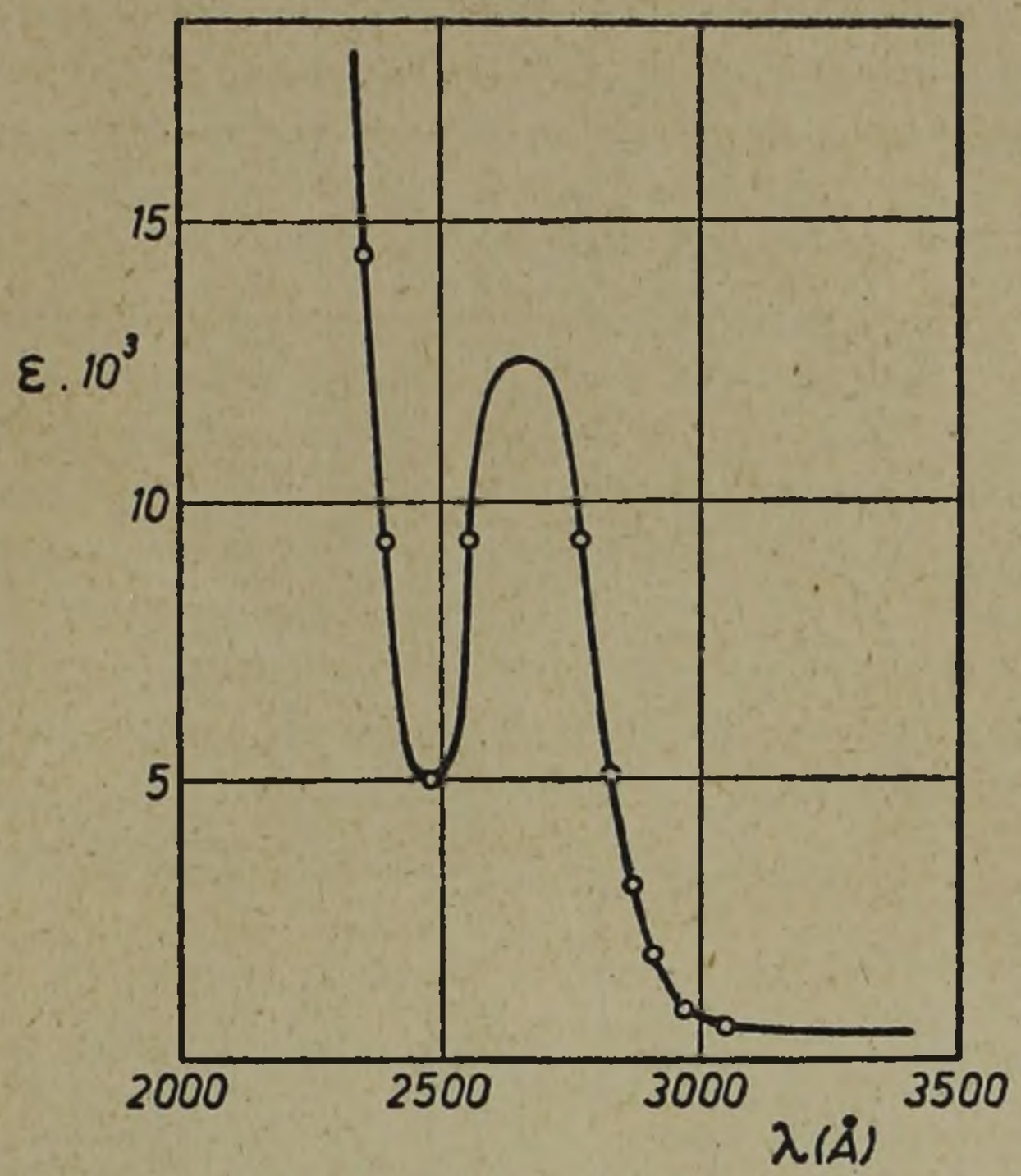

Fig. 1. Absorption spectrum of actin solution.

TABLE II.

Preparation	Ca	Mg
	found as % of dry weight	
Acetone dried muscle I.	0,010	0,048
,, ,, ,, II.	0,010	0,070
Actin solution 1.	0,235	0,041
2.	0,24	
3.	0,25	
4.	0,22	
5.	0,16	
6.	0,23	0,006
7.	0,19	
8.	0,19	
9.	0,22	
Ca content of actin, average	0,215%	

In view of the above findings we have studied the adsorption of cations to actin on the alkaline side of the isoelectric point. We have found in ultrafiltration experiments, that at *pH* 7 there is no difference between the adsorption of *K, Na, Ca* or *Mg* ions. These experiments

could be performed only at ion concentrations where many times more *Ca* is bound to the protein than originaly present in it. Thus they do not throw any light upon the mode of binding of the first few atoms of *Ca*.

EFFECT OF IONS ON THE POLYMERISATON OF ACTIN.

It has already been described that nearly any ion is able to polymerise actin to the same end product. During the study of the effects of the physiologically most important cations, specific differences were found. First, it was found that a small amount of *Mg* ions greatly influences the speed of polymerisation, whereas the effect of *Ca* seems to be inhibitory (cited by *Szent-Györgyi* (3)). These effects are demonstrated in Fig. 2. and Fig. 3., where the polymerisation of actin was studied in a solution resembling the ion concentration of Ringer's solution and in a solution similar to the ionic milieu of the muscle fiber.

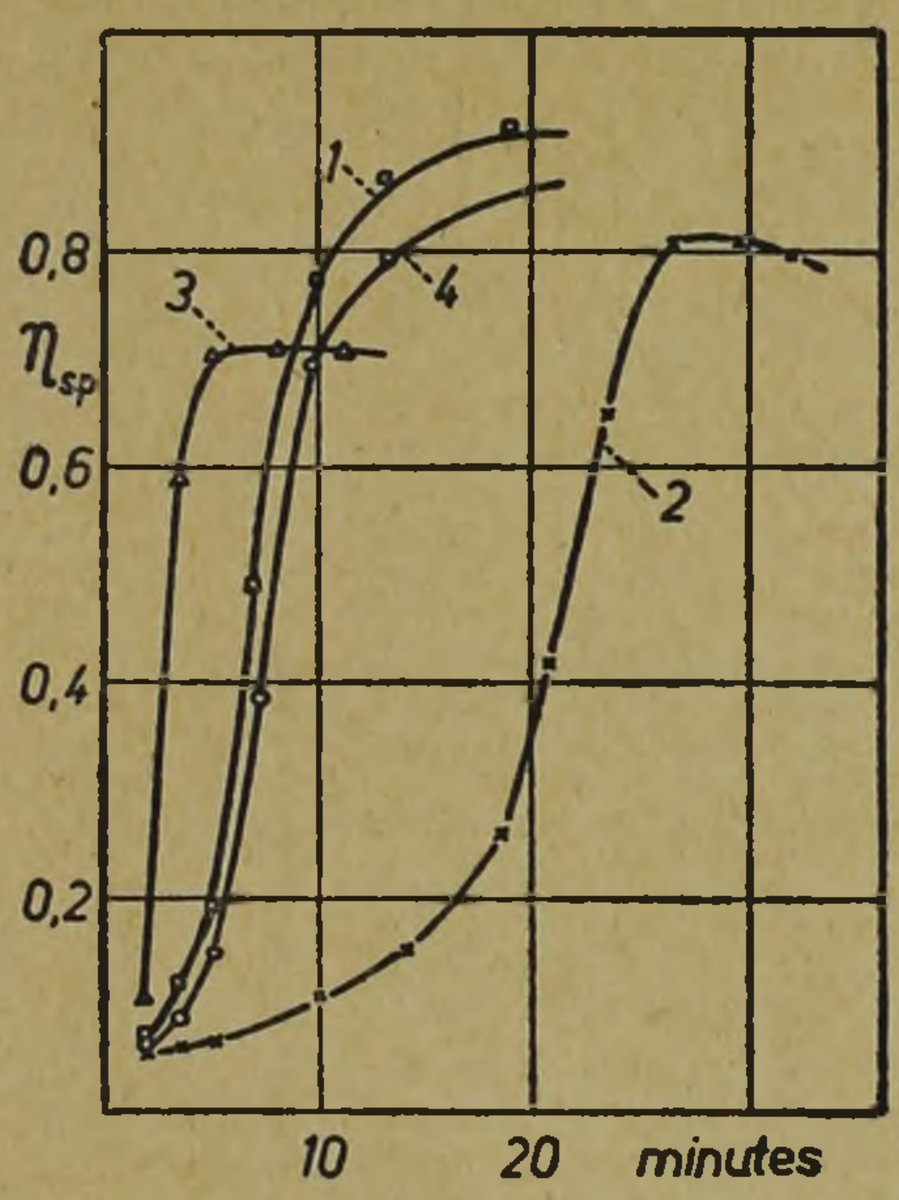

Fig. 2. Polymerisation of actin in presence of various ions. Curve 1: 0,11 *M NaCl*, 0,003 *M KCl*, 0,003 *M* $CaCl_2$, 0,01 *M* $MgSO_4$. Curve 2: same as 1 but without *Mg*; curve 3: same as 1 but without *Ca*; curve 4: same as 1 but without *K*. Temperature 24°.

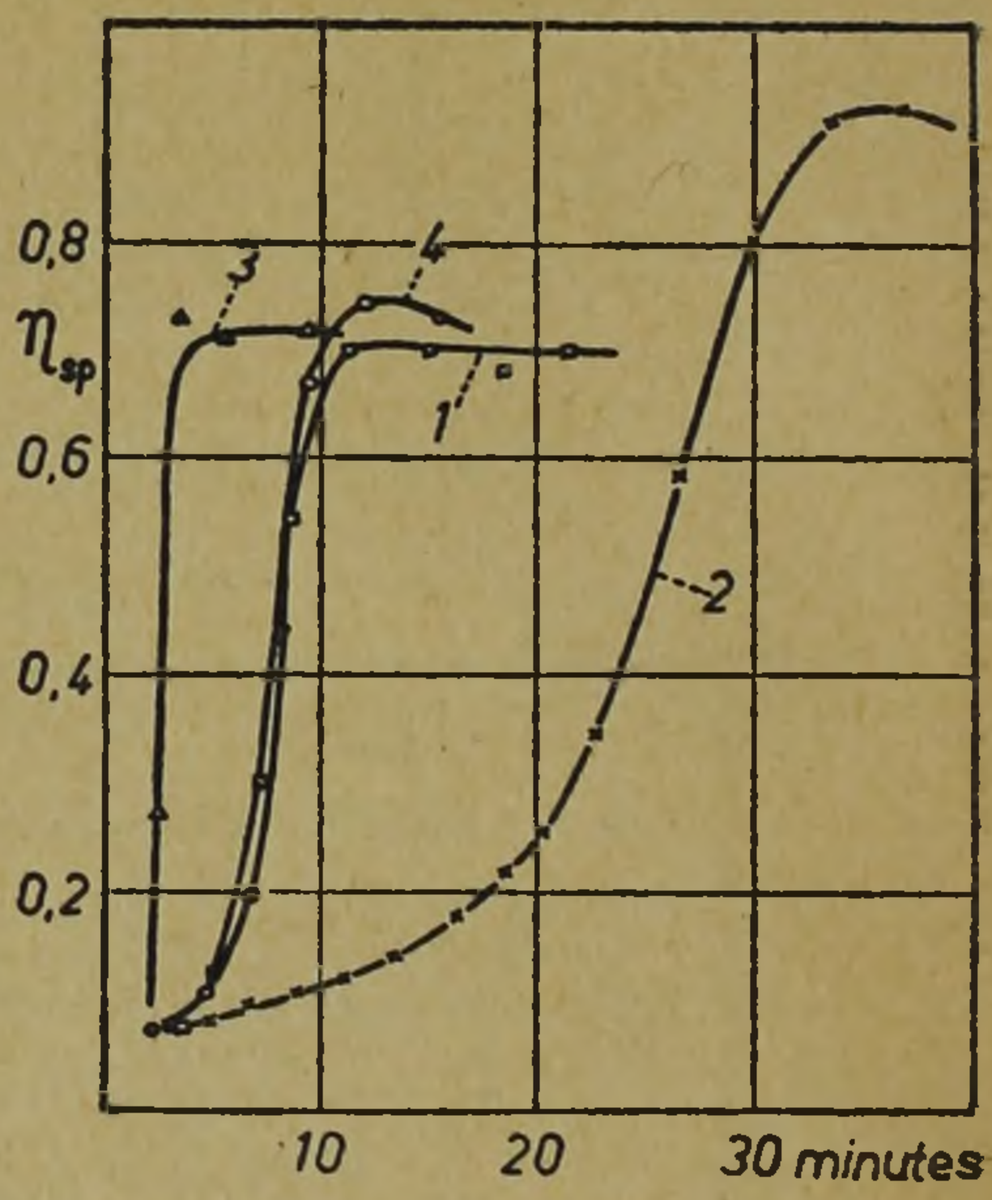

Fig. 3. Polymerisation of actin in presence of various ions. Curve 1: 0,11 *M KCl*, 0,003 *M NaCl*, 0,003 *M* $CaCl_2$, 0,01 *M* $MgSO_4$. Curve 2: same as 1 but without *Mg*; curve 3: same as 1 but without *Ca*; curve 4: same as 1 but without *Na*. Temperature 24°.

There seems to be no difference between the action of *Na*- and *K* ions. *Ca* retards the process of polymerisation in all cases, whereas *Mg* has a pronounced catalytic effect.

We have not yet formed any conclusion about the curious effect shown in Fig. 2. and 3., that in some of the experiments (particularly in those, where *Ca* is present) the viscosity of the actin is for a time higher than the viscosity value at which it becomes finally stabilised. It seems, as if actin would be able to polymerise even to a higher degree, than we have hitherto observed, i. e. giving a higher apparent viscosity than $\eta sp/mg\ N/ml = 1{,}7$. This higher polymerisation product seems to be unstable under the experimental conditons. The *final* value of viscosity attained, is however, very constant for the actin preparations as described in this paper and is independent of the salt used or of the *pH* between *pH* 6—8.

The time curve of the polymerisation reaction is characteristic of autocatalytic reactions. But the polymerisation of actin is not really autocatalytic, as the final product of the polymerisation, polymerised actin, does not catalyse the polymerisation.

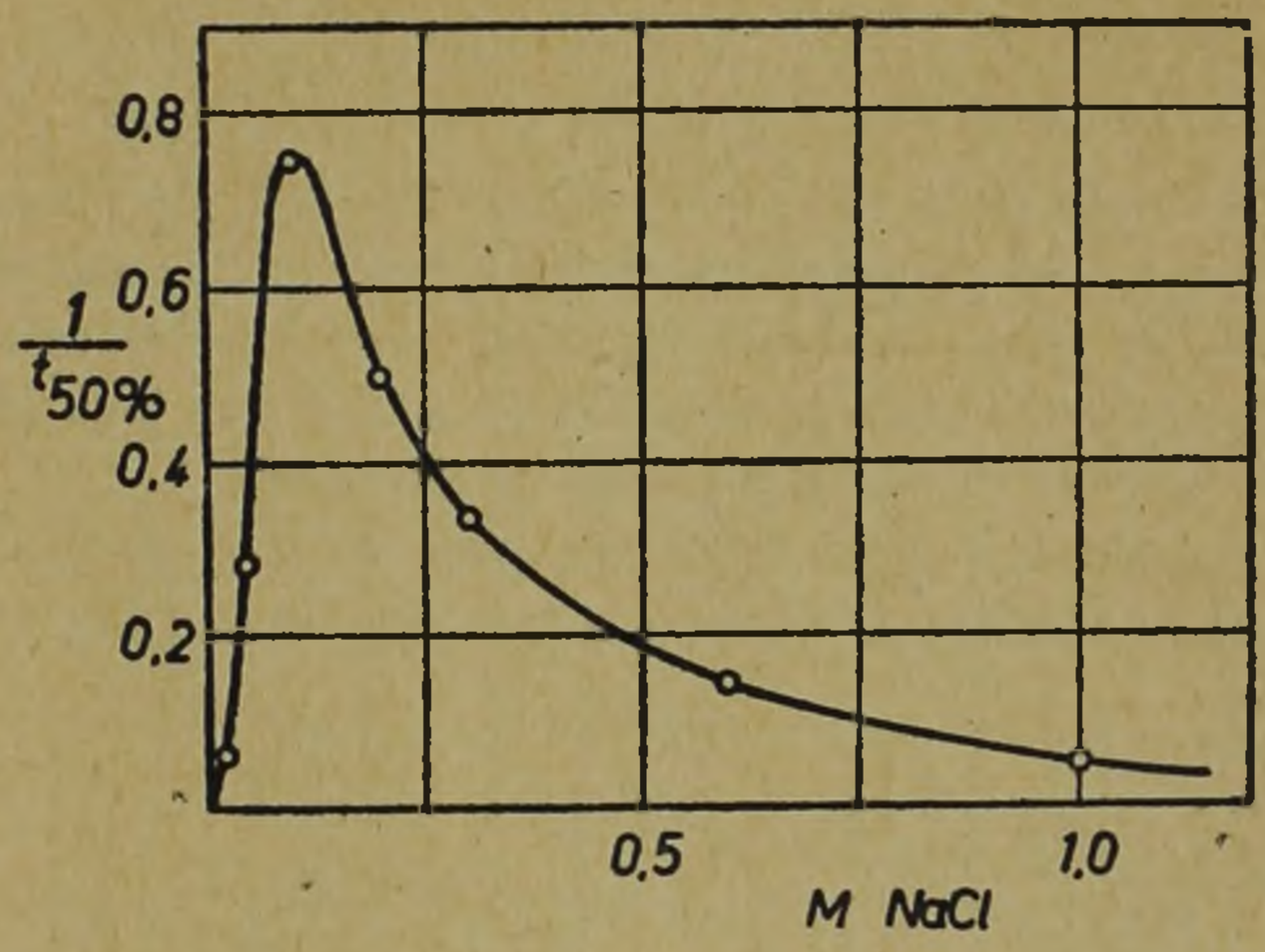

Fig. 4. Variation in the rate of polymerisation in presence of increasing concentrations of *NaCl*. The ordinate denotes the reciprocal value of the time in minutes for 50% polymerisation. Temperature 24 C°.

If we study separately the action of different ions on the rate of polymerisation, the following results are obtained.

The rate of polymerisation under the influence of monovalent cations *(K* and *Na)* has a maximum between 0,1—0,15 *M* concentration. This is shown in Fig. 4. The effect of *Mg* on addition of this ion to 0,1 *M Na* or *K* is shown in Fig. 5.

The effect of *Mg* ions is obviously not so much an increase in the velocity of the polymerisation (i. e. the slope of the rising part of the

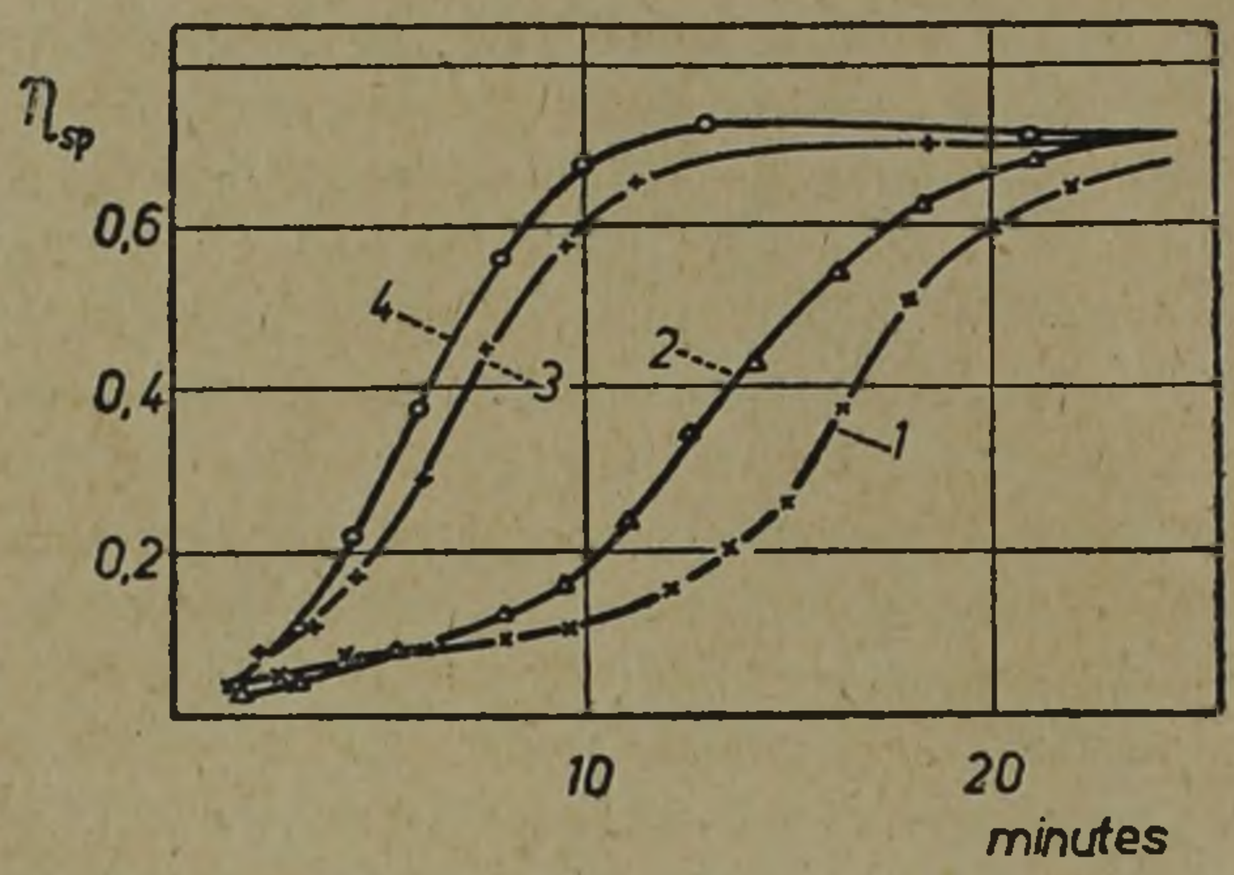

Fig. 5. Polymerisation of actin in presence of 0,1 *M NaCl* with varying concentration of *Mg*. The concentration of added *Mg* is for curve 1: 0, for curve 2: 0,0002 *M*, for curve 3: 0,0005 *M*, for curve 4: 0,001 *M*. Temperature 24 C°.

curve), its main effect is to reduce the time lag, observed when there is no *Mg* present. Actually if we compare the reciprocal value of the time required to reach 50% polymerisation with the amount of *Mg* present (taking into consideration the small amount of ions present in the actin preparation), we come to the conclusion that *Mg* is essential for the polymerisation of actin; without *Mg* there is no polymerisation at all. The slow reaction observed in absence of added *Mg* ions is due to the divalent ions present in the actin preparation itself.

From the above experiments, we came to the conclusion that the polymerisation is a series of reactions, resulting in the overall picture of

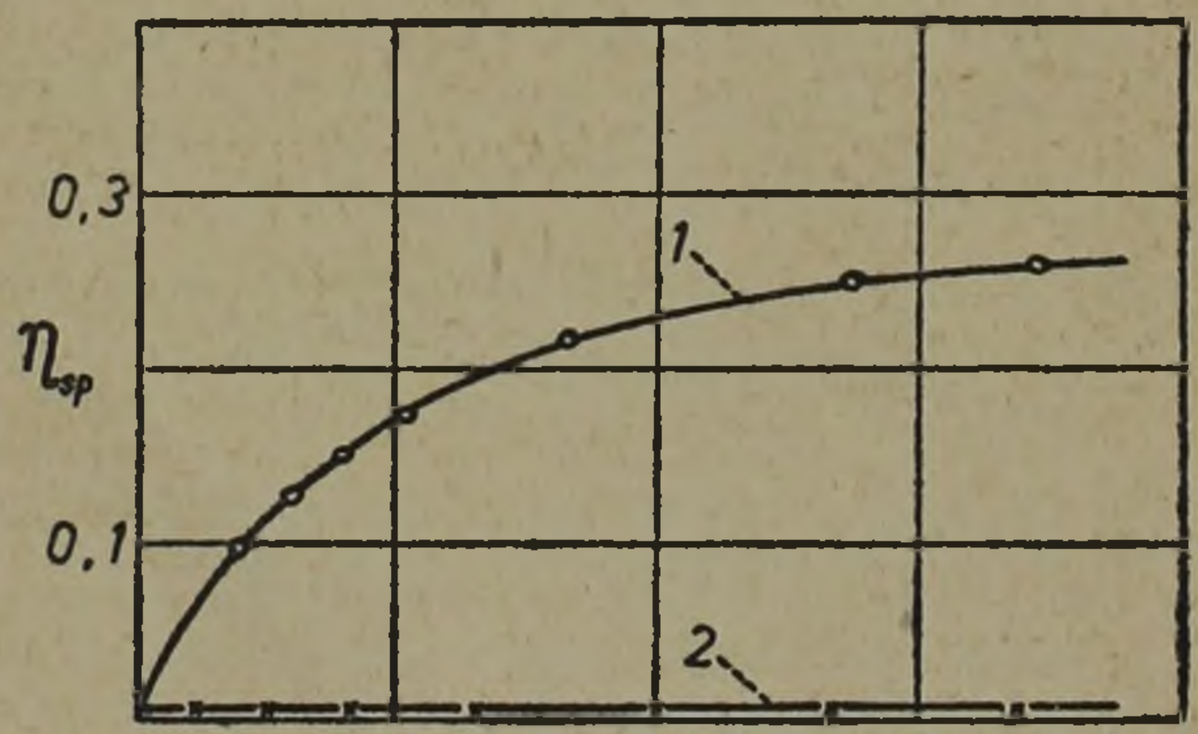

Fig. 6. Curve 1: Actin incubated at 24 C° for 30 minutes with 0,00025 *M* $MgSO_4$. After the addition of 0,1 ml 1% calgon to 4 ml actin solution, it was incubated at the same temperature for further 10 minutes. At time 0, addition of 0,1 *M KCl*. Curve 2: the same amount of actin incubated first with 0,1 ml 1% calgon, then *Mg* added to 0,00025 *M*, kept at 24 C° for 30 minutes, then at time 0 addition of 0,1 *M KCl*.

an autocatalytic reaction and we suppose that *Mg* exerts its influence on the first of these reactions. The experiments recorded in Fig. 6. are in support of this theory. The actin was first incubated with a very small amount of *Mg*, which in itself does not bring about any significant rise in the viscosity. After some time calgon was added to fix the *Mg*, followed by 0,1 *M KCl*. The rate of polymerisation was monomolecular. If calgon is added before *Mg* at the beginning of the experiment, there will be no polymerisation at all on addition of *KCl*. Thus it is evident, that in the first case *Mg* has changed something in the actin, a change, which was not accompanied by any appreciable increase in the viscosity of actin, a change, however, without which *KCl* is unable to effect the polymerisation of actin.

That *Ca* inhibits the rate of polymerisation, is only partially true. We have found that it does so only in presence of monovalent ions like *K* or *Na*. If *Ca* alone is added, it exerts a catalytic influence just like the same amount of *Mg*. It is thus only a matter of choice to say that *Ca* inhibits the rate of polymerisation in presence of *K* or to say that *K* inhibits the polymerisation of actin in presence of *Ca*. This is obvious from the

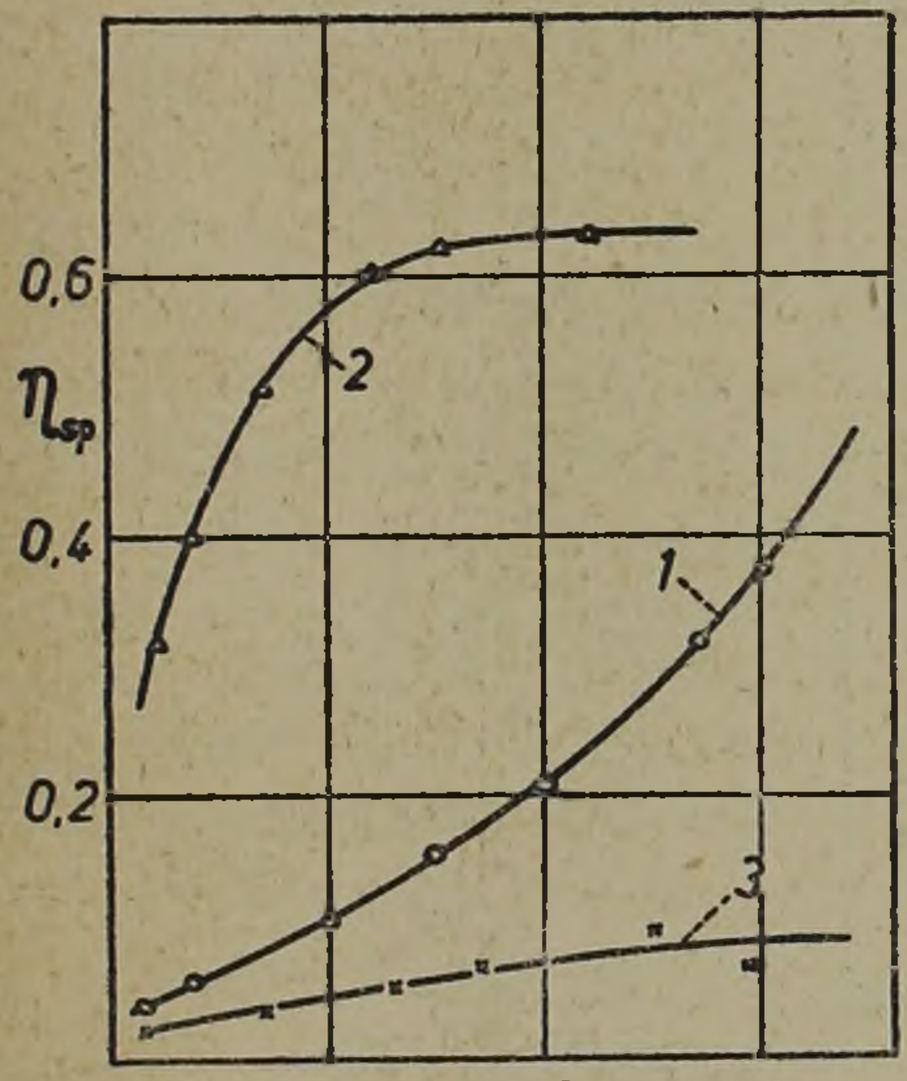

Fig. 7. Polymerisation of actin at 0 C°. Curve 1: in presence of 0,1 *M KCl;* curve 2: in presence of 0,005 *M* $CaCl_2$*;* curve 3: in presence of 0,1 *M KCl* and 0,005 *M* $CaCl_2$.

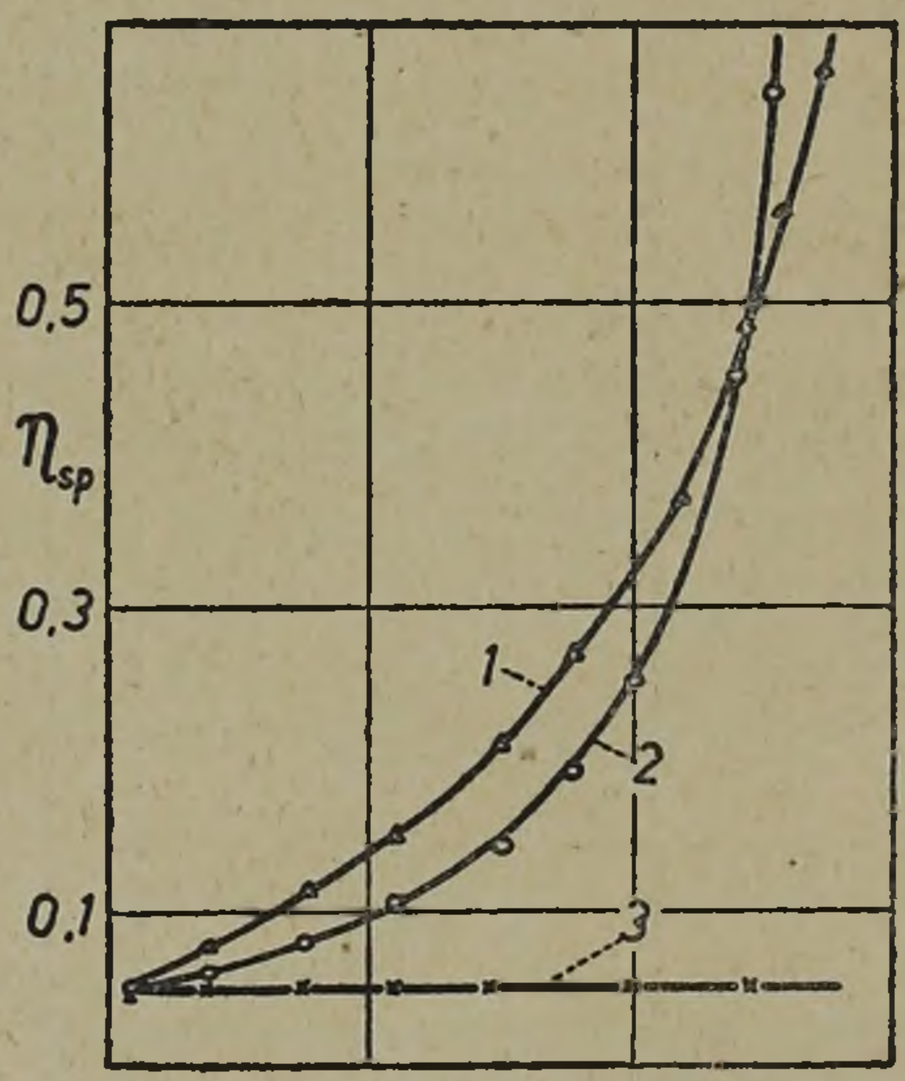

Fig. 8. Polymerisation of actin at 0 C°. Curve 1: in presence of 0,004 *M* $CaCl_2$*;* curve 2: in presence of 0,1 *M NaCl;* curve 3: in presence of 0,004 *M* $CaCl_2$ and 0,1 *M NaCl*. In all three experiments 0,001 *M* $MgSO_4$ was present.

curves of Fig. 7. The ion antagonism observed is not influenced by the presence of *Mg*, as shown in Fig. 8.

REDUCING GROUPS OF ACTIN.

Addition of an oxidizing agent prevents the polymerisation of globular actin, it even destroys polymerised actin. If the oxidation was not too drastic, the effect is reversible: on addition of a reducing substance, polymerised actin is formed again.

Oxidized actin is less stable than native actin, e. g. when globular actin is carefully oxidised, it is destroyed by heat treatment below 40 C^0, whereas native globular actin is stable at 50 C^0 for the same period of time.

While high concentrations of ferricyanide or quinone are needed to oxidize actin, cystine and methylene blue react with actin in relatively dilute solutions. Thus a 2 mg/ml actin solution is partly reversibly oxidised by 0,1 *M* ferricyanide, 0,01 *M* *p*-benzoquinone, 0,001 *M* cystine and the same concentration of methylene blue.

If a small amount of $KMnO_4$ is added to an actin solution, this prevents the polymerisation reaction; it can be used to stop the polymerisation at a desired moment. The effect is reversible. Addition of more

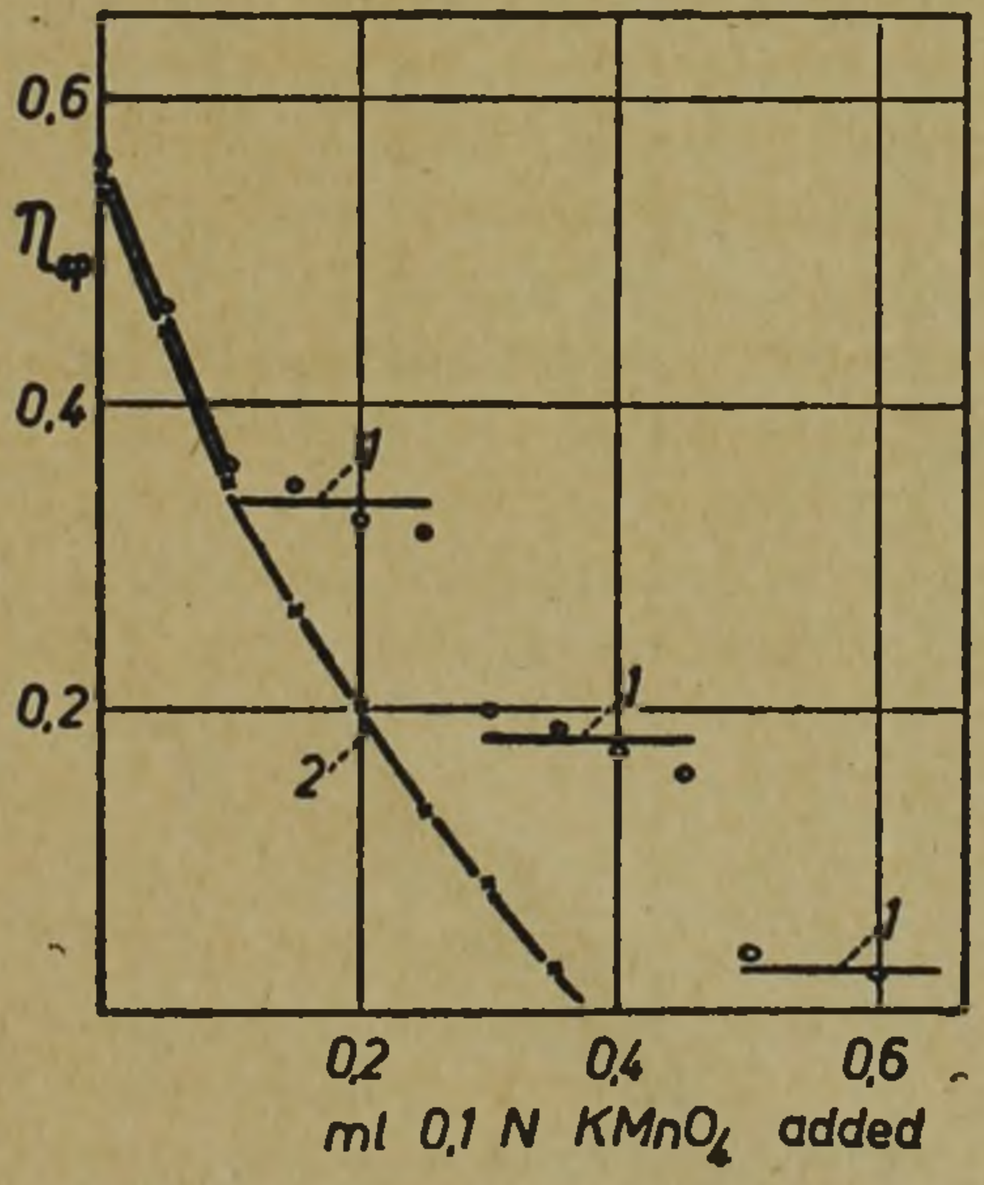

Fig. 9. The effect of increasing amounts of $KMnO_4$ on the viscosity of polymerised actin. Curve 1: actin polymerised in presence of 0,1 *M KCl;* curve 2: actin polymerised in presence of 0,1 *M KCl* and 0,005 *M* $MgSO_4$.

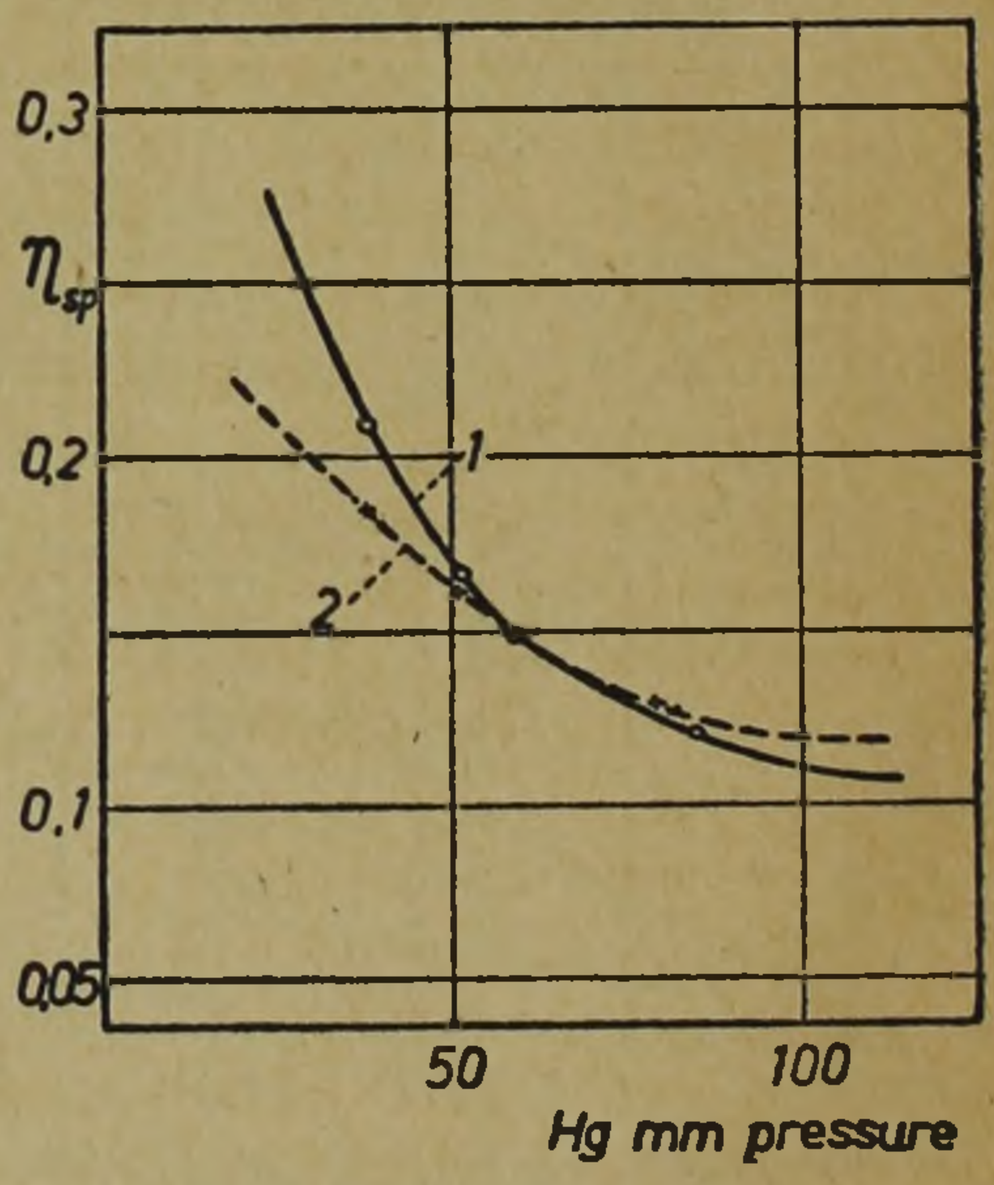

Fig. 10. The effect of pressure on the apparent viscosity of actin. Curve 1: dilutep polymerised actin ,untreated. Curve 2: 5 ml polymerised actin solution treated with 0,4 ml 0,1 *N* $KMnO_4$.

$KMnO_4$ destroys even po ymerised actin. It is very interesting to note that if actin is titrated with increasing amounts of $KMnO_4$ a step-wise degradation of polymerised actin is observed (Fig. 9., curve 1). We have observed the same 3 steps with various actin preparations.[1]

Fig. 10 shows the relation of specific viscosity to the pressure applied in the viscometer, for two different actins: the solid line was obtained with the original polymerised actin, the dotted line with actin partially oxidised by the addition of 0,4 ml of 0,1 N $KMnO_4$ to 5 ml actin. (The original actin solution was diluted for the measurement so as to give the same apparent viscosity at $p = 60$ Hg mm as the undiluted oxidised actin.) It is seen that the apparent viscosity of the partially oxidised actin is less dependent on the pressure, than that of the untreated polymerised actin. In other words it consists of shorter particles.

It is obvious from the above experiments that the polymerisation involves such groups which are easily oxidized by cystine, methylene blue, etc. It has to be supposed moreover, that the forces joining together the major units are more accessible to the oxidant than those linking together the primary or minor units, i. e. that 3 or 4 different reactions lead to the final polymerisation product.

The stepwise degradation of polymerised actin is observed in those cases, where the polymerisation was brought about by the addition of monovalent ions only. If the polymerisation was effected by 0,1 M KCl in presence of 0,005 M $MgSO_4$, a continuous breakdown is observed, as represented in curve 2, Fig. 9.

We want to mention in this connection the earlier observation that addition of Mg ions has a pronounced influence on the thixotropy of actin solutions. In absence of Mg ions the apparent viscosity — decreased by high shearing forces — returns during rest to its original higher value but slowly, while in presence of Mg ions the original value is reached much faster. This means that the recombination of major units is brought about by Mg ions.

From this and from the above experimental findings we conclude, that Mg ions combine with the oxidizable groups and thus they, as well as the oxidizable groups take part in joining together particles of globular actin into particles of fibrous actin. Mg ions stabilise the polymerisation products with regard to mechanical forces, whereas at the same time their presence makes these groups more accessible to oxidizing agents.

[1] The effect of $KMnO_4$ in these experiments is irreversible. The viscosity of actin at the level of the first step does not change on standing, whereas that of actin at the second step gradually decreases in time.

Experiments to establish the nature of the oxidizable groups are still in progress.

PROSTHETIC GROUP OF ACTIN.

If a globular actin solution is dialysed against distilled water or against a very dilute bicarbonate solution, it gradually loses its ability to polymerise on addition of salts, with a parallel loss of ability to form actomyosin. This loss of activity may be prevented by dialysing the actin solution against a solution of boiled actin or against a diluted boiled muscle juice.

Similar results may be obtained by washing the isoelectric precipitate of actin with dilute acetate buffer solution. If the actin solution is diluted to contain about 0,05 mg protein/ml, precipitated at pH 5, washed 2—3 times with the same volume of 0,01 *M* acetate buffer of pH 5, the precipitate finally dissolved in dilute bicarbonate, a more or less turbid solution is obtained, which will not polymerise on addition of salts, neither will it be able to form the viscous actomyosin, when added to myosin.

The loss of activity is prevented if a dilute solution of boiled actin or boiled muscle juice is added to each washing. The extent to which the native properties of actin are retained, depends on the quantity of boiled juice added. This is shown in Fig. 11, representing a typical experiment. The amount of actin present after 3 washings with acetate buffer,

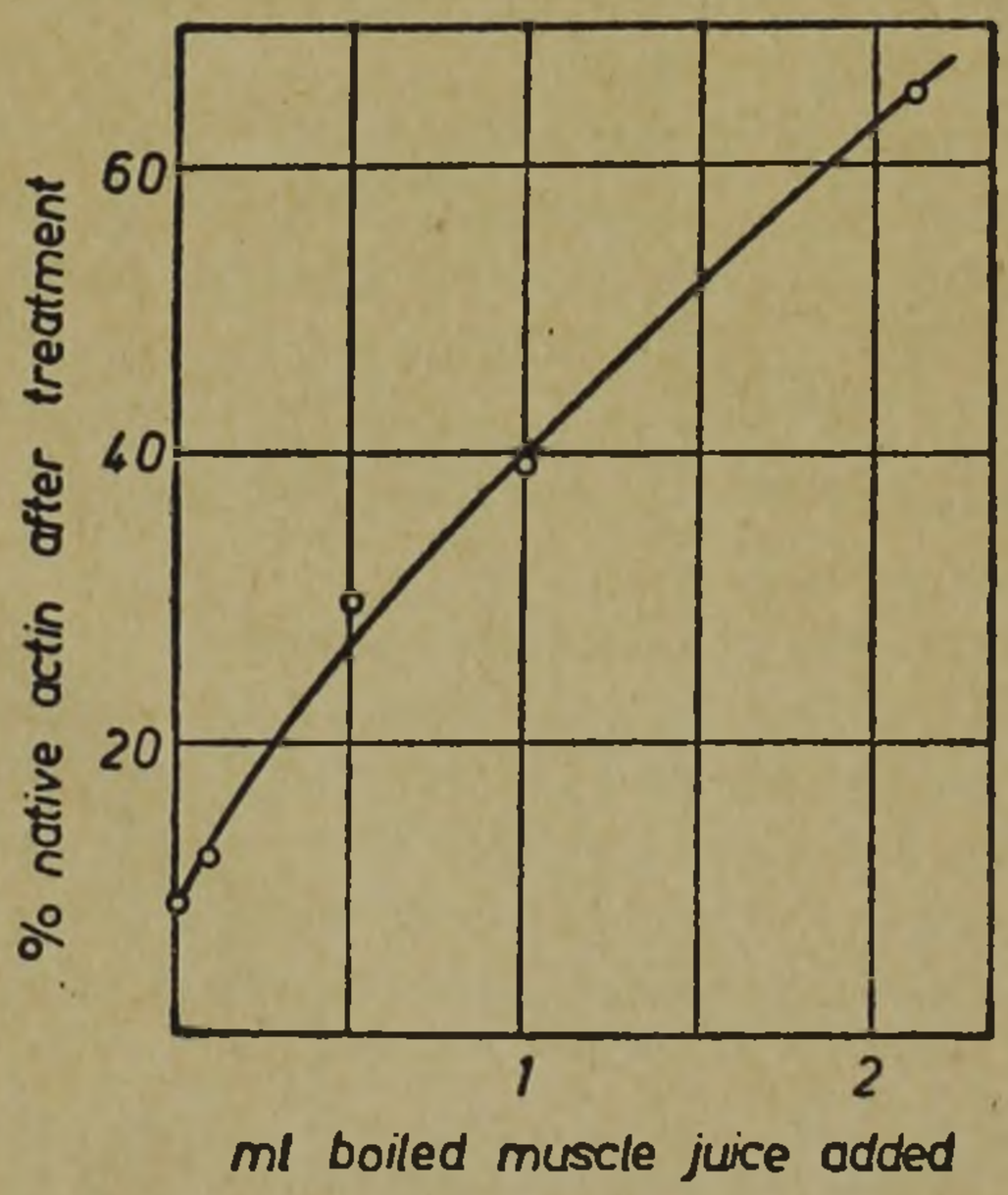

Fig. 11. Effect of boiled beef heart muscle extract (1 : 2) on isoelectrically washed actin.

was determined according to the method of *Straub* (2) by studying its reaction with myosin.

We have not yet succeeded in obtaining a preparation of actin, which is in itself inactive towards myosin, and which on addition of boiled juice would revert to the native, active actin. Thus the nature of the factor, present in the boiled muscle juice cannot be decided yet. It may be a stabilising impurity, the presence of which prevents the denaturation of actin, or it may be a real prosthetic group. There are many examples known in enzyme chemistry — not to mention the simple case of hemoglobin — where the removal of the prosthetic group makes the protein many times more labile. This may explain why we were not able to obtain the free protein component in the native state.

In accordance with our experiments on the oxidizability of actin, we find that if a small amount of reducing substance is added during the above procedure of isoelectric washing, the removal of our factor is slowed down. However, the factor itself is not a reducing substance. The chemical nature of the factor is now under investigation.

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CONTRIBUTIONS TO THE MODE OF ACTION OF PENICILLIN

(WITH 5 FIGURES IN TEXT.)

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The correlation of bacterium metabolism and the mode of action of antibiotic substances has been the object of several reports. These reports are, regarding penicillin, in accordance when stating that this antibiotic substance acts upon living microorganisms only. In present experiments the oxygen-consumption of staphylococci has been examined with Warburg's technique, further, with Thunberg's method, the dehydration rate of glucose and the donators preexisting in the bacteria; both kinds of experiments having been performed under the influence of penicillin and without this influence. *Werner* (1) found in 1943 on the basis of similar experiments that the effect exercised by raw penicillin preparations on the respiration of bacterium suspensions was composed of two phases: 1. increase of respiration, and 2. impeded respiration leading after some hours to abolishing of respiration. The author assigned an importance only to the second phase. In his view the first phase were caused by notatin, an enzym probably identical with the glucose oxydase. This ferment being invariably present in raw filtrates is produced by the Penicillium notatum. The pure preparations are free of this ferment, wherefore respiration is never increased by them.

In our experiments 24 hours old agar cultures of staphylococci were employed. The colonies had been washed with 0,9 p. c. sodium chloride, centrifuged and twice washed each with 30 ml water, then weighed in moist condition, finally suspended in a physiologic solution containing also PO_4 buffer in a final concentration of m/15. Thus a *pH* of 7,3 was maintained. As to the bacterium content of the suspension, 3 ml of latter contained 30 mgr. bacteria. This suspension exhibited for several hours a marked oxygen consumption in Warburg's apparatus even when no glucose or other nutriment had been added. The addition of penicillin resulted, independently of its concentration, in the biphasic reaction reported on by *Weber*. The phenomenon could invariably be observed,

independently of the extent to which the penicillin preparation had been purified (Fig. 1). The oxygen consumption of the bacteria influenced by penicillin increases whether or not glucose has been added to the suspension. As it can be seen from the figure the absolute order of value of this initial increase remains unchanged after glucose addition. We have inferred from these data that penicillin effect consists in the oxidation taking place during the first phase of a specific constituent of the bacterium.

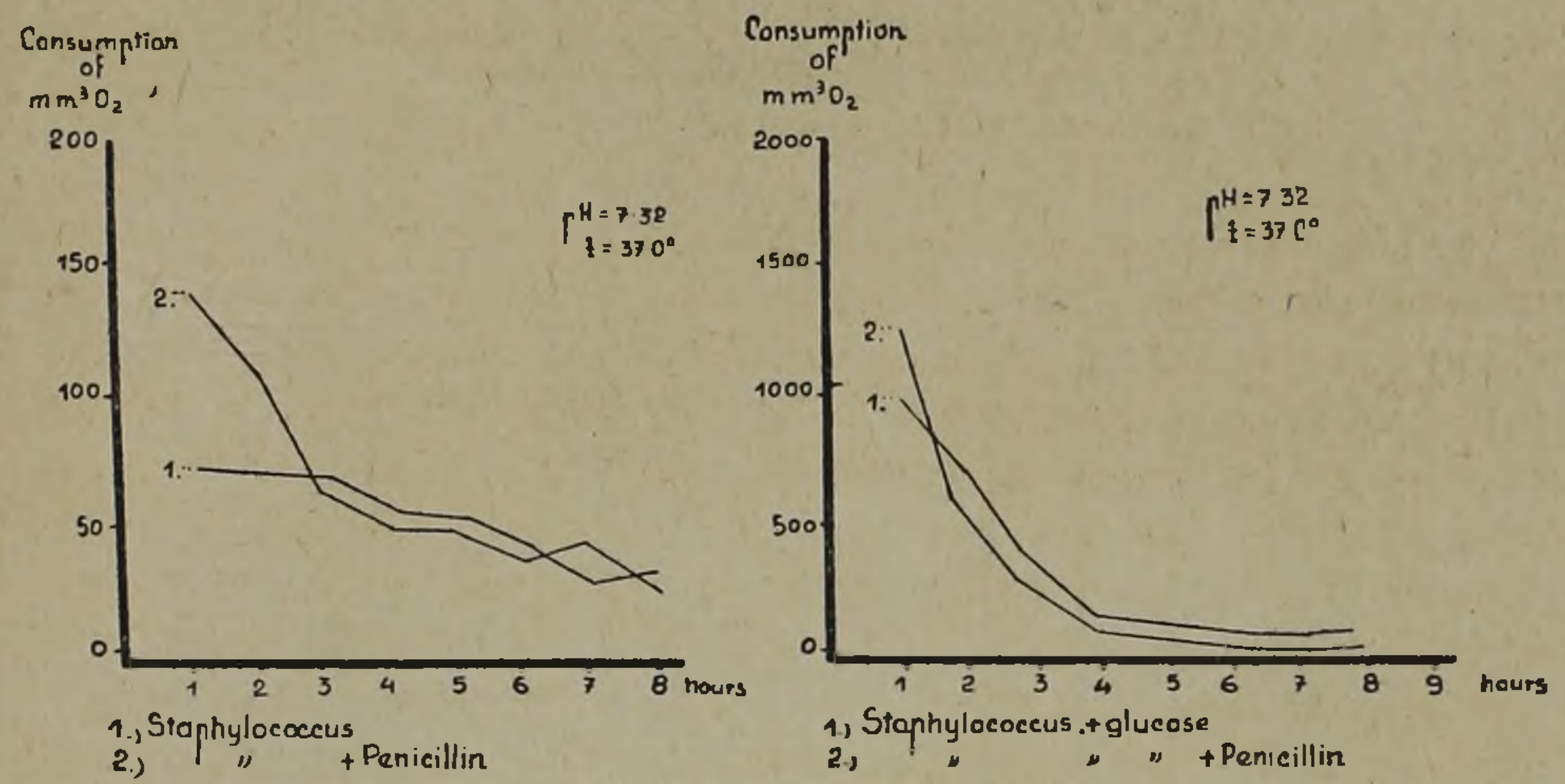

Fig. 1.

This specific oxidation manifests itself in an increase of oxygen consumption. We do not believe that this phenomenon be due to Werner's glucose oxydase, because in latter case the increase of oxygen consumption ought to be much more considerable in a suspension containing glucose than in the suspension free of glucose.

The staphylococcus suspension employed in our experiments does, under anaerobic conditions, decolorate methylene blue with a high speed. The time of reduction is longer in the presence of penicillin[1] (Fig. 2). This phenomenon is by no means due to the paralysis of bacterium dehydrase the bacteria displaying in Warburg's apparatus, in the presence of penicillin, even after 7 or 8 hours a marked oxygen consumption pointing to an intact dehydrase function. The reduction of methylene blue in Thunberg's apparatus can be modified by varying the concentration of the bacterium suspension: the more concentrated the suspension, the greater the impeding action of penicillin, and conversely, with increasing dilution

[1] In these experiments the purest penicillin available, the product CSC of the Commercial Solvents Corporation, was used.

the impeding action gradually diminishes until it turns into stimulation (Fig. 3.). This strange mechanism still wants of a closer examination. No doubt, the impeding action of penicillin cannot be explained otherwise than by its capacity of playing the role of a hydrogen acceptor. Very likely the affinity of penicillin to the substance to be dehydrated is considerably stronger than that of the methylene blue. On the other hand, it has been proved that the dehydration of penicillin is a reversible process,

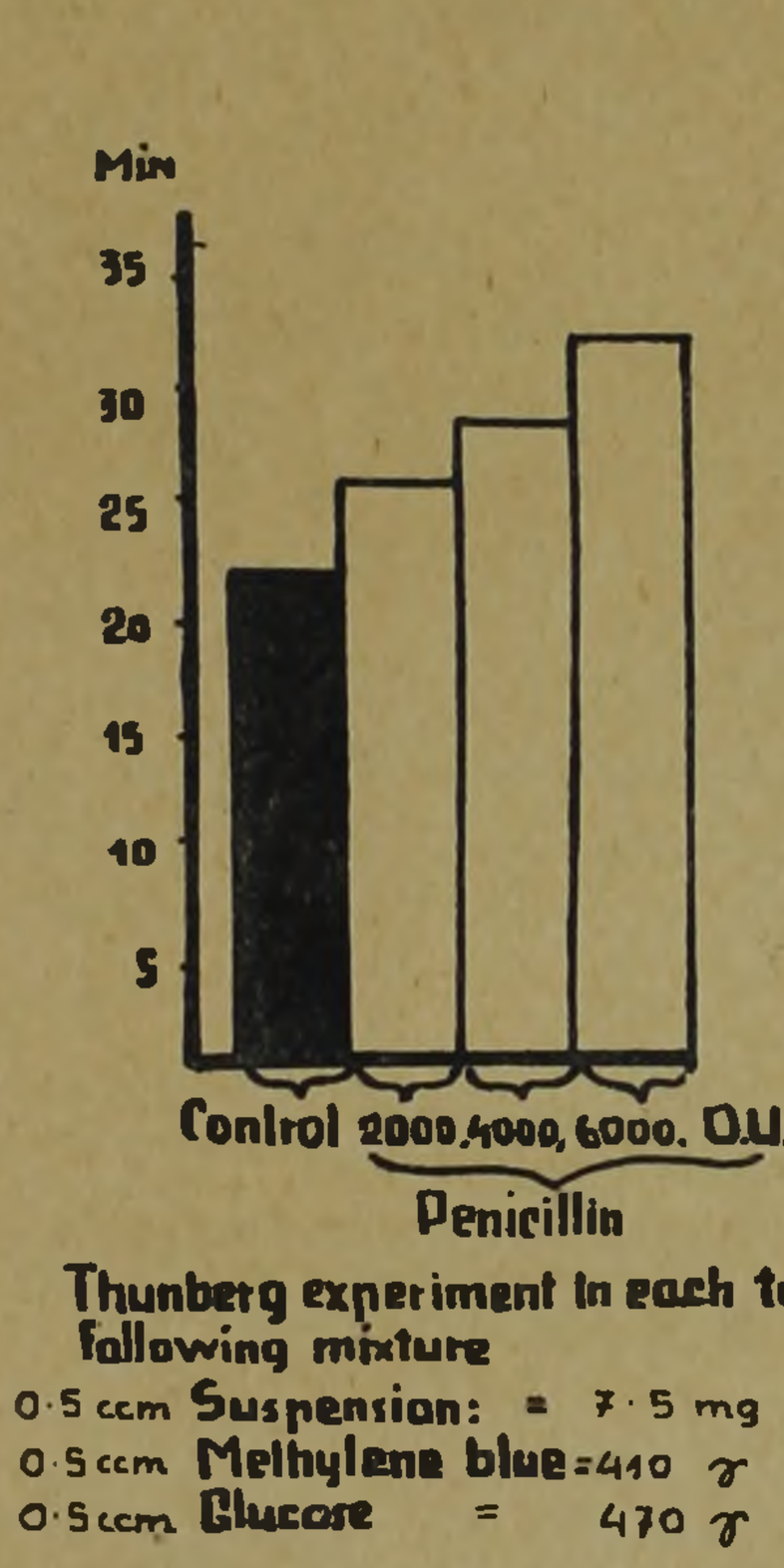

Fig. 2.

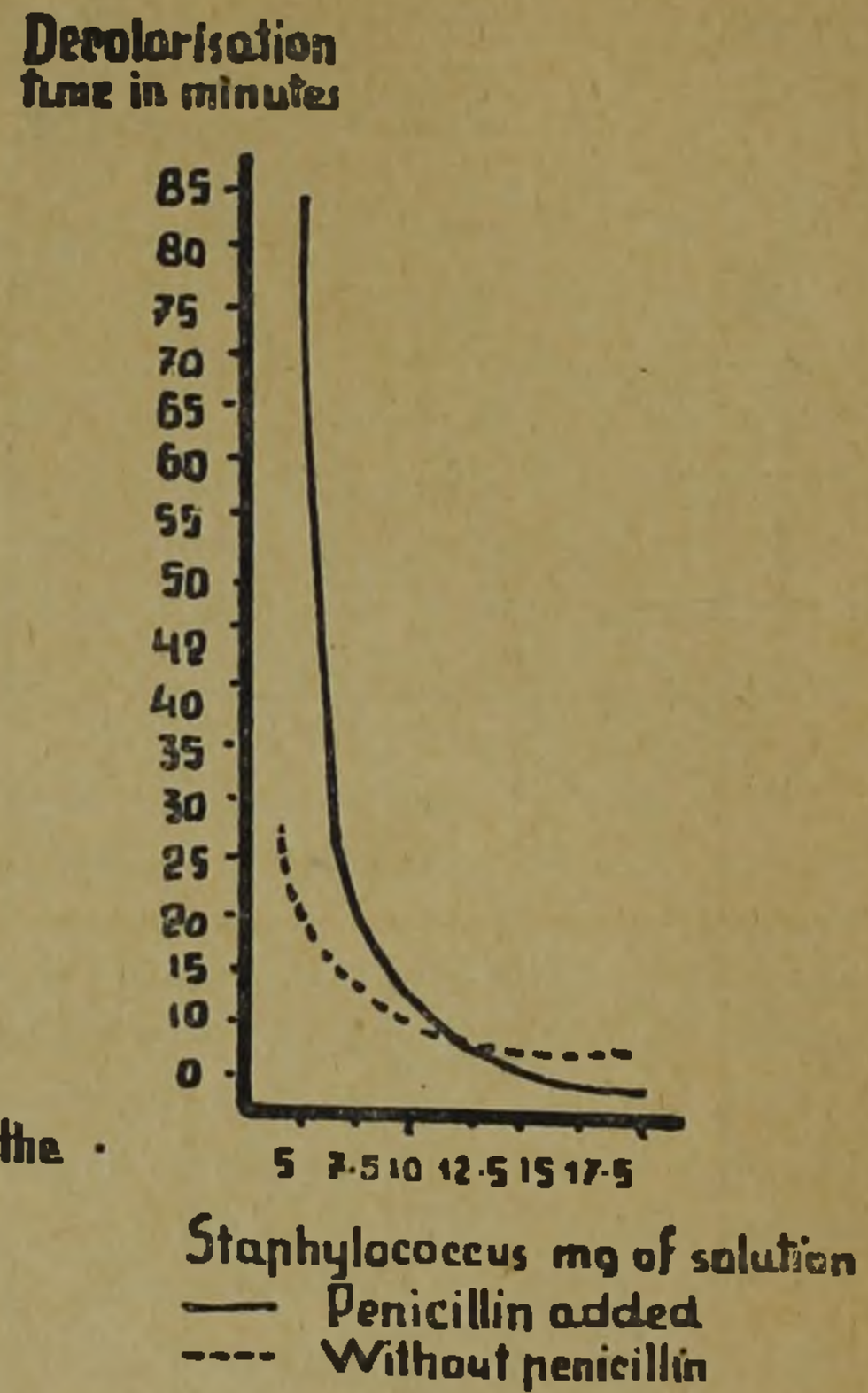

Fig. 3.

that is no chemical decomposition takes place in its molecule in the course of its antibiotic action *(Hobby Chaffee* and *Mayer* — 2, 3). Mayer could regain the substance, after the penicillin effect was over, with unaltered efficacy.

The fact that it is the dehydrogenating oxidation which is influenced by penicillin has been proved in experiments in which the methylene blue reduced by staphylococcus suspension became recolorised in the presence of minimum quantities of penicillin. Similar reactions occur

only with substances lending themselves to reversible hydration and dehydration. When re-oxidation of methylene blue leukobasis by penicillin took place in Borsook's apparatus by means of which the potential can be determined during the process the observation was made that the potential underwent sudden changes toward the positive direction of the redox scale (Fig. 4.). Thus the penicillin effect results in oxidative processes ensuing in the bacterium suspension. These oxidative processes have no

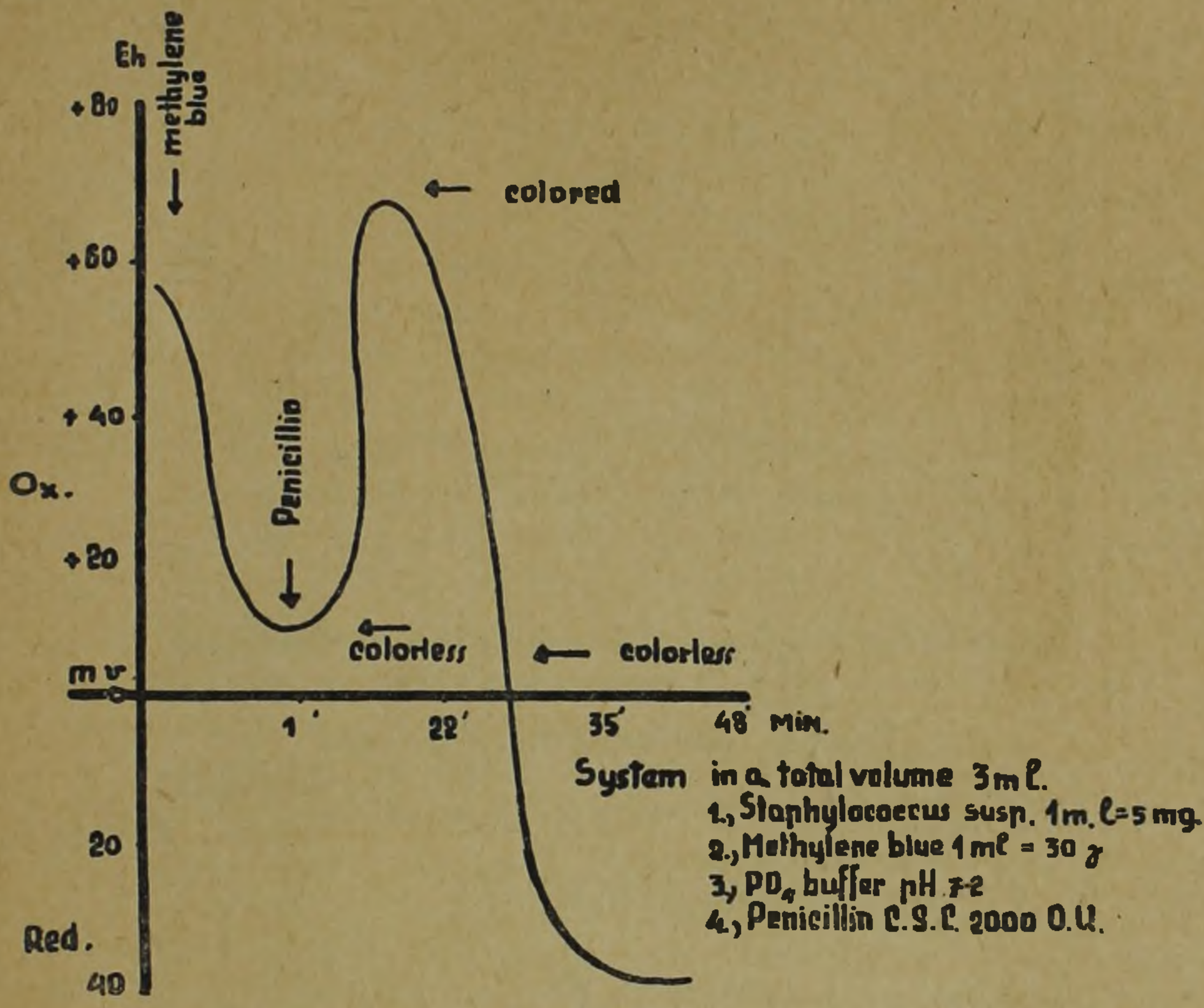

Fig. 4.

influence upon the individual metabolism of the bacteria they do, however, have a trend against a stimulating agent of bacterium proliferation.

The main goal of further observations was to obtain data referring to the portal of entrance where penicillin injures the microorganisms. Washing of the staphylococci for a long time yields, as Quastel has stated, bacteria which, though alive, consume minimal quantities of oxygen and do not develop. We too developed such „resting" bacteria and obtained following results with them: they consume nearly no oxygen, they fail to decolorise methylene blue in Thunberg's apparatus. If they are added

glucose as a source of energy an oxygen consumption takes place the value of which exhibits little variety. If they are added, beside glucose, the washing fluid used before and concentrated in vacuum a respiration curve showing striking deviation from those obtained hitherto comes about: under the influence of this fluid termed activator a gradual increase of the oxygen consumption takes place. The activator completes the action of glucose whereby the proliferation of the bacteria ensues,

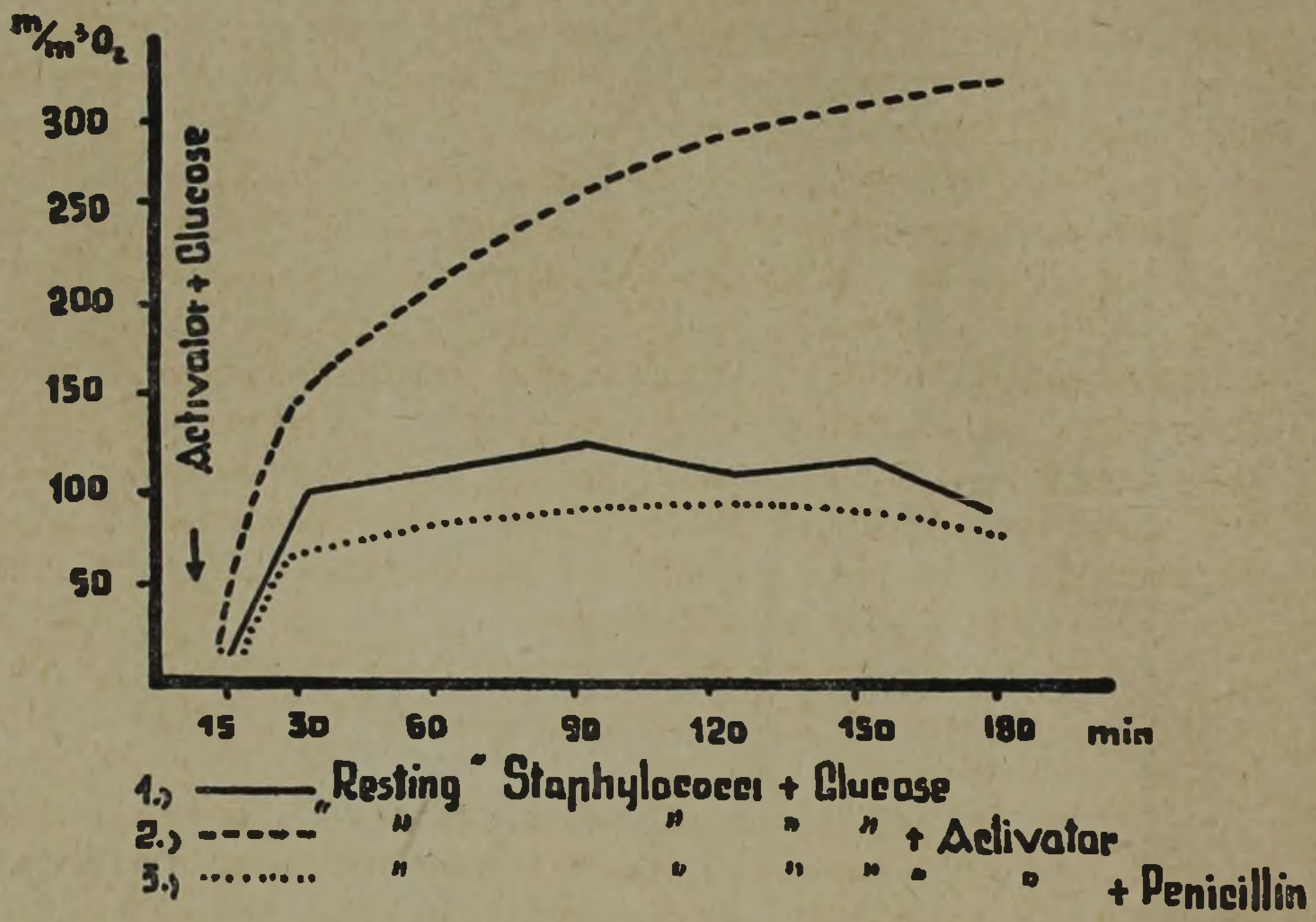

Fig. 5.

beside their individual metabolism. No doubt, the washing fluid contains an agent called activator inducing bacterium proliferation. Present experiments do not permit any conclusion as to whether this substance belongs to the type p-amino-benzoic acid or to that of pantothenic acid. As yet, nothing more has been revealed than its thermostability and sensitivity to arsenic. It was evident that the correlation of the activator to penicillin was to be examined (Fig. 5.). In the experiments the effect of the activator upon the resting bacteria was abolished by penicillin. Experiments done with Thunberg's method supplied a further support for these data. This observation is in full accordance with the data of the literature (4) that is penicillin abolishes bacterium proliferation without influencing their respiration.

Further observations could be performed with the activators of a washing fluid of staphylococcus cultures which had been cultivated for a longer time in artificial media. These activators proved less efficacious than those produced of the medium of the first passage. Apparently, the activator forming property is, parallel to the adjustment to artificial media, gradually decreasing.

It has been mentioned above that we are ignorant of the activator content of the fluid containing this substance. Obviously, the substance inducing bacterium proliferation belongs neither to the type of p-aminobenzoic acid nor to that of panthothenic acid because, as stated by *Werner*, and C. *Levaditi* (5), neither of these compounds can neutralise the action of penicillin. Nevertheless, the fact that penicillin can paralyse the so-called logarithmic phase of bacterium proliferation has given rise to the assumption that it is the ferment system arranging the proliferative phase which is impaired by penicillin bringing about alterations in the oxidative and reductive processes. At this item a resemblance to the observations of *Jeney* (6) should be pointed to who found that certain spectrum fields of the ultra violet section exert their impeding effect only upon the logarithmic phase of bacterium proliferation. Similar impairment can be observed with the bacteriophages influencing the proliferation also at this sensitive phase.

All these phenomena seem to prove that the chemical factor promoting bacterium proliferation is very sensitive to changes of the oxidative and reduction processes.

Our results may be summarized in following: 1. The respiration of staphylococci is stimulated by penicillin in the first period of its action, and is slightly impaired later. 2. The oxido-reduction equilibrium experiences, under penicillin action, a shift toward the oxidations. 3. The factor inducing proliferation with resting bacteria can be paralysed by penicillin.

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